Sustainable Agriculture Reviews 35

Grégorio Crini
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Sustainable Agriculture Reviews 35

Chitin and Chitosan: History, Fundamentals and Innovations

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Sustainable agriculture is a rapidly growing field aiming at producing food and energy in a sustainable way for humans and their children. Sustainable agriculture is a discipline that addresses current issues such as climate change, increasing food and fuel prices, poor-nation starvation, rich-nation obesity, water pollution, soil erosion, fertility loss, pest control, and biodiversity depletion.

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Editors

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Chitin and Chitosan: History, Fundamentals and Innovations

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Preface

*Je vais faire connaître la nature de la substance qui forme le corps ou la base charnue insoluble du champignon, et que je désignerai sous le nom de fongine**
November 15, 1810 – The discovery of chitin
Professor Henri Braconnot
Professor of Natural History
Director of the Botanical Garden of Nancy, France

**I will make known the nature of the substance that forms the frame or the insoluble fleshy part of mushrooms, and I will name this substance fongine*

Professor Henri Braconnot (1780–1855). (Source: Simonin F. Notice biographique sur M. Henri Braconnot. Compte Rendus des Travaux de la Société de Médecine de Nancy, 1834–1855 (1856) pp. 51–79)

Most commercial polymers are actually derived from petroleum-based raw products using chemical processes, which are not always safe and environmental friendly. Over the past three decades, there has been a growing interest in developing natural alternatives to synthetic polymers, namely, biopolymers. Biopolymers

are polymers derived directly from living organisms or synthetized from renewable resources. Biopolymer production has been growing steadily due to their biodegradability and absence of toxicity. Biopolymers include polysaccharides such as chitin and chitosan. Chitosan is produced by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans, such as crabs and shrimp, and cell walls of fungi. Due to their remarkable macromolecular structure, physical and chemical properties, and bioactivities, chitin and chitosan have received much attention in fundamental science, applied research, and industrial biotechnology.

This book, *Chitin and Chitosan*, is the first volume of two volumes published in the series Sustainable Agriculture Reviews. Written by 57 international contributors from 21 different countries, who are leading experts in the chitin and chitosan field, these volumes focus on the developments, research trends, methods, and issues related to the use of chitin and chitosan for both fundamental research and applied technology.

The first volume provides an overview of history, fundamentals, and innovations of chitin and chitosan. For newcomers to the field, the book starts by a chapter on historical landmarks in the discovery of chitin, by Grégorio Crini, followed by fundamentals and applications of chitosan in the second chapter by Nadia Morin-Crini et al. In Chapter 3, Swati Jaiswal et al. review the biocatalytic production of hetero-chitosan oligosaccharides as antioxidant. Enzyme immobilization on chitin and chitosan-based supports for potential biotechnological applications are then presented by Madan L. Verma et al. in the fourth chapter. Chapter 5 by Tadashi Uragami discusses the application of chitin and chitosan derivative membranes in resources, energy, environmental, and medical fields. Chitosan for 3D printing and bioprinting is then reviewed by Thomas J. Kean and Maya Thanou in Chapter 6. Yoshihiko Hayashi discusses the contribution of D-glucosamine to cell membrane stability in Chapter 7. The last chapter by Daniele Massella et al. describes manufacturing techniques of chitosan-based microcapsules to enhance the functional properties of textiles. The second volume covers the applications of chitin and chitosan in food, agriculture, pharmacy, medicine, and wastewater treatment.

The editors extend their thanks to all the authors who contributed to this book for their efforts in producing timely and high-quality chapters. The creation of this book would not have been possible without the assistance of several colleagues and friends deserving acknowledgment. They have helped by choosing contributors and reviewing chapters and in many other ways. Finally, the editors would like to thank the staff of Springer Nature for their highly professional editing of the publication.

Besançon, France — Grégorio Crini
Aix-en-Provence, France — Eric Lichtfouse

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About the Editors

Dr. Grégorio Crini, 52, is researcher at University Bourgogne Franche-Comté, Besançon. His current interests focus on the design of novel polymer networks and the environmental aspects of polysaccharide chemistry. He published over 190 papers in international journals and books and is a highly cited researcher. The total citation of his publications is over 9000, h-index of 33. https://www.researchgate.net/profile/Crini_Gregorio

Dr. Eric Lichtfouse, 59, is a biogeochemist at Aix Marseille University who has invented carbon-13 dating, a molecular-level method allowing to study the dynamics of organic compounds in temporal pools of complex environmental media. He is chief editor of the journal *Environmental Chemistry Letters* and the book series Sustainable Agriculture Reviews and Environmental Chemistry for a Sustainable World. He is the author of the book *Scientific Writing for Impact Factor Journals*, which includes an innovative writing tool: the micro-article. https://cv.archives-ouvertes.fr/eric-lichtfouse

Chapter 1
Historical Landmarks in the Discovery of Chitin

Grégorio Crini

Abstract In 1799, Hatchett decalcified shells of crabs, lobsters, prawns, and crayfish with mineral acids, observing that "they produced a moderate effervescence and in a short time were found to be soft and plastic of a yellowish color and like a cartilage, which retained the original figure". Although this is the first mention of calcified chitin in invertebrates, the discovery of chitin is usually attributed to Braconnot in 1811 who discovered it from fungi, and its recognition to Odier in 1823 who obtained a hornlike material after treatment of cockchafer elytra with potassium hydroxide. Chitin first named *fongine* by Braconnot and then *chitine* by Odier. Children revealed the nitrogenous nature of chitin in 1824. The history of chitosan, the main derivative of chitin, dates back to 1859 with the work of Rouget. The name of chitosan was, however, introduced in 1894 by Hoppe-Seyler. In 1876, Ledderhose hydrolyzed arthropod chitin and discovered *glykosamin*, the first derivative of chitin. The main aim of this chapter is to describe the 220 years of the development of chitin. I have roughly divided it into five periods: discovery from 1799 to 1894, a period of confusion and controversy from 1894 to 1930, exploration in 1930–1950, a period of doubt from 1950 to 1970, and finally the period of application from 1970 to the present day. The different periods are illustrated by considering examples of studies that appeared in the literature and in particular those of several great scientists who have left their mark on the history of this polysaccharide. Although this historic review cannot hope to be exhaustive, it does highlight the work of those researchers who have contributed to the development of our knowledge of chitin throughout the 220 years of its history.

Keywords Chitin · Chitosan · History · Discovery · Braconnot · Controversy · Exploration · Period of doubt · Period of application

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1.1 Introduction

Chitin is the most abundant of the renewable polysaccharides in the marine environment and one of the most abundant on Earth after cellulose. Cellulose is the significant structural polymer in the primary cell walls of the plant cells while chitin is the main structural polymer found in the fungal cell wall. Chitin is also present on exoskeletons of arthropods and insects. Cellulose and chitin are both β-1,4 linked polysaccharides composed respectively of glucose and N-acetylglucosamine, i.e. 2-acetamido-2-deoxy-D-glucose. These two polysaccharides are used industrially in many different applications. Because of their similarity, research on chitin and cellulose has often been interwoven, with reciprocal advantages, e.g. studies on constitution, chemistry, biosynthesis, and mostly (micro-fibrillar) structure.

The history of chitin began in France, in the beginning nineteenth century, with the work of the chemist Henri Braconnot. Braconnot in 1811 named the insoluble residue remaining after the extraction of fungi with water, alcohol, and dilute alkali *fongine*/fungine (Braconnot 1811a, b). He discovered chitin polysaccharide from fungi, preceding cellulose by about 30 years. However, chitin was probably discovered by Charles Hatchett, an English chemist, who, during his experiments on the shells of marine animals, reported in 1799 the presence in the cuticle of "a material particularly resistant to usual chemical" (Hatchett 1799). However, there is no indication that he was aware of what he had done.

The history of chitosan, the main derivative of chitin, dates back to 1859 with the work of Charles Rouget who reported that treatment of *chitine* with concentrated caustic potash solution under reflux gave a new "*chitine modifiée*", modified chitin, and this treatment rendered it soluble in organic acids (Rouget 1859). The name of chitosan was, however, introduced in 1894 by Felix Hoppe-Seyler for an acid-soluble derivative of chitin prepared from the treatment of the shell of crabs, scorpions and spiders (Hoppe-Seyler 1894).

This chapter reviews the main historical landmarks in the discovery and the development of chitin, and also chitosan, its main derivative, reported during the period 1799–2018. I have roughly divided it into five periods: discovery from 1799 to 1894, a period of confusion and controversy from 1894 to 1930, exploration in 1930–1950, a period of doubt from 1950 to 1970, and finally the period of application from 1970 to the present day. The different periods are illustrated by considering examples of studies that appeared in the literature and in particular those of several great scientists who have left their mark on the history of these polysaccharides.

1.2 Discovery: 1799–1894

The first period, from 1799 to 1894, covers the discovery of chitin and chitosan. The names that marked this period most were Charles Hatchett, Henri Braconnot, Auguste Odier, John George Children, Charles Rouget, Georg Ledderhose, and Felix Hoppe-Seyler.

1.2.1 Charles Hatchett

Charles Hatchett (1765–1847) was an English chemist and a self-formed mineralogist and analytical chemist (Johnson 1803; Good et al. 1813; Smedley et al. 1845; Wisniak 2015). In 1799, Hatchett decalcified shells of crabs, lobsters, prawns, and crayfish with mineral acids. In a 20-page memory, Hatchett described a material particularly resistant to usual chemical (Hatchett 1799). In June 13, 1799, he wrote that "it appears that immersion of the shell in acetous, or in dilute nitric acid, afforded carbonate and phosphate of lime, the former, however, in largest quantity". This is the first mention of calcified chitin in invertebrates (Smedley et al. 1845). In February 1800, in the Journal of Natural Philosophy, Chemistry, and the Arts (Fig. 1.1), Hatchett wrote that "Pieces of this fubftance, taken from various parts of thofe animals, was at different times immerfed in acetous and in diluted nitric acid; thofe which had been placed in the diluted nitric acid produced a moderate effervefcence, and in a fhort time were found to be foft and elaftic, of a yellowifh white colour, and like a cartilage, which retained the original figure" (Hatchett 1800a, b). However, Hatchett did not push his scientific investigations any further, preferring to become a noted collector of books, paintings, musical instruments, and musical manuscripts (Wisniak 2015).

1.2.2 Henri Braconnot

Henri Braconnot (1780–1855) was a French chemist, Director of the *Jardin Botanique* of Nancy (Fig. 1.2). His work was essentially devoted to the extractive principles of natural vegetables and various aspects of the chemistry and physiology of natural substances including carbohydrates and alkaloids. Braconnot's scientific career covers the period 1806 to 1854, in which he published over 110 *mémoires* (Simonin 1856, 1870; Nicklès 1856a, b; Donzelot 1953; Prévost and D'Amat 1956; Labrude 1997; Labrude and Becq 2003; Wisniak 2007; Nwe et al. 2011; Muzzarelli et al. 2012).

In 1811, Braconnot discovered an alkaline-insoluble fraction from fungi such as *Agaricus volvaceus*, by treatment with dilute warm alkali (Braconnot 1811a, b, c). He gave it the name of *fongine* (fungine), a substance "*d'une nature particulière*"

JOURNAL

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AND

THE ARTS.

FEBRUARY 1800.

Experiments and Obſervations on Shell and Bone. By CHARLES HATCHETT, *Eſq. F. R. S.* *

SOME experiments, which I lately made at the requeſt of Mr. Home, and which he has done me the honor to mention in his ingenious paper on the teeth of granivorous quadrupeds, induced me to turn my attention more particularly to the chemical examination of ſhell and bone, eſpecially as the former appeared to have been hitherto much neglected.

The time ſince theſe experiments were began, has not been ſufficient to enable me to enter into all the minutiæ of the chemical analyſis of theſe ſubſtances; but as ſome remarkable facts were aſcertained, I have now ventured to bring them forward, with the addition of ſome obſervations, although as yet the whole is little more than a very imperfect outline.

The firſt of theſe experiments were made on the ſhells of marine animals; and to avoid repetition and prolixity, I ſhall, in a great meaſure, once for all, deſcribe the menſtrua, the precipitants, and the mode of operation.

When ſhells were examined they were immerſed in acetous acid, or nitric acid diluted, according to circumſtances, with 4, 5, 6, or more parts of diſtilled water; and the ſolution was always made without heat.

Fig. 1.1 Extract of the article by Hatchett on his experiments and observations on shell and bone in February 1800. (Hatchett 1800a)

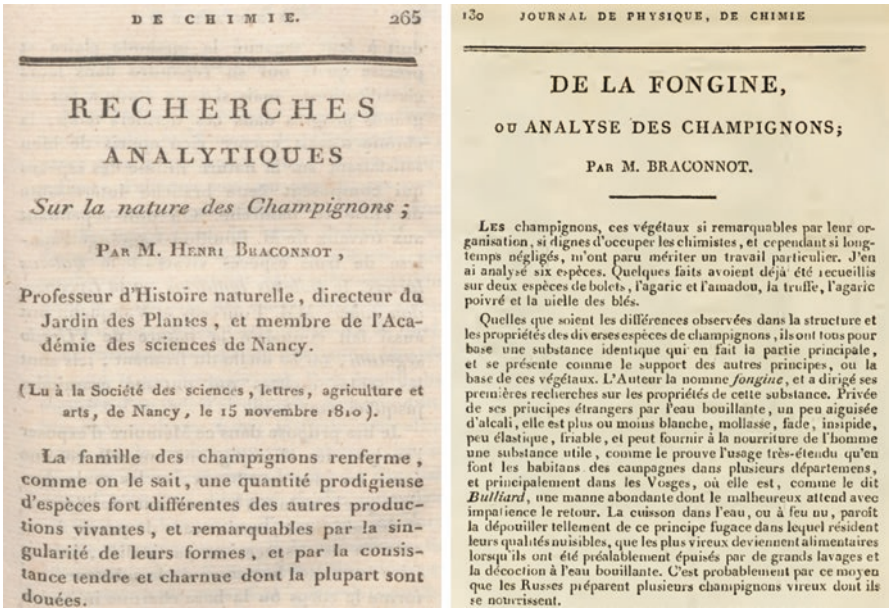
(Fig. 1.3). Braconnot analyzed the nitrogen content in the liquid obtained by distillation of this fraction, and found that the liquid contained acetate of ammonia contaminated with oil. This fraction also produced acetic acid by degradation with concentrated sulfuric acid (Braconnot 1811b). In a later paper, Braconnot observed that his *fongine* had many different consistencies, more or less soft, leathery, cartilaginous, or cork-like, indicating that there were differences in the proportion of its components (Braconnot, 1813). He repeatedly stated that his *fongine* differed from woody materials, containing more nitrogen than wood but much less than wheat protein or in animal materials.

Fig. 1.2 Professor Henri Braconnot, 1780–1855. (Source: Simonin 1856)

1.2.3 Auguste Odier

In August 17, 1821, Auguste Odier presented a *mémoire* at the *Société d'Histoire Naturelle de Paris* on a new substance found in the elytra of insects (Fig. 1.4), published in 1823 (Odier 1823; Bounoure 1919). Odier isolated an insoluble alkaline-insoluble fraction from cockchafer (May beetle) by repeated treatments with hot caustic potash solutions. He gave it the name of *chitine*, from Greek word chiton ("*χιτών*"), meaning a covering, tunic or envelope (Odier 1823; Straus-Durckeim 1828; Payen 1843). After several treatments, Odier observed that *chitine* was stable in external form and soluble in sulphuric acid "with the assistance of heat" but did not become yellow by the action of nitric acid. However, *chitine* was dissolved when digested in it with heat. At the same time, a similar substance named *entomaderme* was also observed by the young student Lassaigne (Straus-Durckeim 1828; Latreille 1831). Odier concluded (1) *"la chitine est une substance particulière, fort curieuse"* and (2) *"il est fort remarquable de retrouver dans la charpente des insectes la même substance qui forme celle des végétaux"*. Indeed, he thought the frameworks of insects and of plants were composed of the same substance, cellulose.

Odier noted that *chitine* made up only a relatively small part of the insect cuticle: the elytra of *Melolontha* contained about 29%. There was a certain amount of ash



DE CHIMIE. 265

RECHERCHES
ANALYTIQUES
Sur la nature des Champignons ;

PAR M. HENRI BRACONNOT,

Professeur d'Histoire naturelle, directeur du Jardin des Plantes, et membre de l'Académie des sciences de Nancy.

(Lu à la Société des sciences, lettres, agriculture et arts, de Nancy, le 15 novembre 1810).

La famille des champignons renferme, comme on le sait, une quantité prodigieuse d'espèces fort différentes des autres productions vivantes, et remarquables par la singularité de leurs formes, et par la consistance tendre et charnue dont la plupart sont douées.

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DE LA FONGINE,
OU ANALYSE DES CHAMPIGNONS;

PAR M. BRACONNOT.

LES champignons, ces végétaux si remarquables par leur organisation, si dignes d'occuper les chimistes, et cependant si longtemps négligés, n'ont paru mériter un travail particulier. J'en ai analysé six espèces. Quelques faits avoient déjà été recueillis sur deux espèces de bolets, l'agaric et l'amadou, la truffe, l'agaric poivré et la nielle des blés.

Quelles que soient les différences observées dans la structure et les propriétés des diverses espèces de champignons, ils ont tous pour base une substance identique qui en fait la partie principale, et se présente comme le support des autres principes, ou la base de ces végétaux. L'Auteur la nomme *fongine*, et a dirigé ses premières recherches sur les propriétés de cette substance. Privée de ses principes étrangers par l'eau bouillante, un peu aiguisée d'alcali, elle est plus ou moins blanche, mollasse, fade, insipide, peu élastique, friable, et peut fournir à la nourriture de l'homme une substance utile, comme le prouve l'usage très-étendu qu'en font les habitans des campagnes dans plusieurs départemens, et principalement dans les Vosges, où elle est, comme le dit *Bulliard*, une manne abondante dont le malheureux attend avec impatience le retour. La cuisson dans l'eau, ou à feu nu, paroît la dépouiller tellement de ce principe fugace dans lequel résident leurs qualités nuisibles, que les plus vireux deviennent alimentaires lorsqu'ils ont été préalablement épuisés par de grands lavages et la décoction à l'eau bouillante. C'est probablement par ce moyen que les Russes préparent plusieurs champignons vireux dont ils se nourrissent.

Fig. 1.3 First page of two *mémoires* of Braconnot published in 1911 left) in the journal *Annales de Chimie* (Braconnot 1811a) where he described his experiments, and right) in the *Journal de Physique, de Chimie, d'Histoire Naturelle et des Arts* where he proposed the name of *fongine* (Braconnot 1811c)

and some oils. The bulk of the non-chitinous substance was considered by him to be protein (Straus-Durckeim 1828; Latreille 1831). Odier also identified his *chitine* as present in demineralized crab carapace and suggested that it was the basic material of the exoskeletons of all insects, and possibly, the arachnids (Odier 1823; Straus-Durckeim 1828; Latreille 1831; Bounoure 1919). He tested for nitrogen in this residue and came to the conclusion that it did not contain nitrogen (Odier 1823). In 1855, Edmond Frémy (1814–1894), another French chemist, reported a similar conclusion for his *chitine/fongine*, named by him *metacellulose* (Frémy 1855). The nitrogenous nature of *chitine* was revealed by the experiments of Children (1824). However, the discovery that nitrogen was present in chitin of insect origin is usually attributed to both Lassaigne (1843a, b, c) and Payen (1843). Both Straus-Durckeim (1828) and Latreille (1831) previously indicated that the presence of nitrogen was highlighted by the young student Lassaigne in *entomaderme* (*chitine*).

MÉMOIRE

SUR

LA COMPOSITION CHIMIQUE

DES PARTIES CORNÉES DES INSECTES.

PAR M. AUGUSTE ODIER.

(LU DANS LA SÉANCE DU 17 AOUT 1821.)

DEPUIS assez long-temps, des expériences souvent répétées nous ont fait connaître la composition chimique des os des vertébrés du premier ordre. Quelques savans, curieux de comparer la charpente osseuse des animaux inférieure à celle des êtres plus élevés dans l'échelle zoologique, ont appliqué leurs connaissances à l'analyse des premiers. C'est ainsi que nous avons appris des chimistes la composition des os des poissons, du test des mollusques, des crustacées et des parties dures des zoophytes.

Mais dans ces divers travaux on n'avait encore jamais pris pour objet de recherches chimiques les parties solides du corps des insectes, et l'on se contentait de les assimiler aux matières animales auxquelles elles ressemblent le plus, soit pour les fonctions, soit pour l'apparence physique : de-là les uns, les comparant aux os

Fig. 1.4 First page of the *mémoire* presented by Odier at the *Société d'Histoire Naturelle de Paris* in August 17, 1821. (Odier 1823)

1.2.4 John George Children

Odier's paper was promptly published in an English translation for the Zoological Journal by Children in 1824 (Fig. 1.5). John George Children (1777–1852) was a British chemist, mineralogist and zoologist at the British Museum. He felt that Odier's conclusion that the *chitine* was nitrogen free because the products of its dry distillation had no effect on test papers was open to debate (Smedley et al. 1845). It is important to note that Children was not aware of Braconnot's much earlier work on *fongine*.

ART. XV.—*Memoir on the Chemical Composition of the Corneous parts of Insects.* By M. AUGUSTUS ODIER.* *With some additional remarks and experiments, by* J. G. CHILDREN, Esq. F.R.S. L.S. &c.

Experiments, often repeated, have long since taught us the chemical composition of the bones of vertebrated animals of the first order. The skeletons of animals of inferior rank in the zoological scale, have also been subjected to chemical analysis, for the purpose of comparison with those of the former, whence we have obtained a knowledge of the component parts of the bones of fishes, the shells of mollusca and crustacea, and the hard portions of the zoophyta.

The solid parts of the bodies of insects, however, have never been made the subject of chemical research; naturalists have been satisfied with likening them to those animal substances which they most nearly resemble either in their functions, or natural appearance; hence some, comparing them to the bones of vertebrated animals, have named them osseous parts, and have even ventured to call the whole assemblage the skeleton of the insects; others, on the contrary, likening them to the integuments of vertebrated animals, have considered them as hardened skin, or a matter analogous to horn.

In undertaking to investigate the chemical composition of these organs, my object is not to support either of these two opinions; I shall examine the substances with the sole view of ascertaining their composition, without seeking to associate them with any particular organ of the superior animals.

The analysis of some *Crustacea*, by M. M. Merait, Guillot, and Chevreul, are the only works that have been published, as far as I know, on animals approaching the class of insects; and amongst these much discrepancy exists as to the composition of their integuments.*

* Translated from the "Memoires de la Societé d'hist. nat. de Paris," vol. I.

† Has M. Odier never heard of Mr. Hatchett's elaborate "Experiments and Observations on Shell and Bone," or his "Chemical Experiments on Zoophyta?" See Phil. Trans. 1799 and 1800. C.

Fig. 1.5 First page of the article of Children where he translated the Odier's paper and described his own experiments in 1824. (Children 1824)

Children, repeating the same experiments, also extracted an alkaline-insoluble fraction from May bug elytra. He observed that "during the action of the alkali, a slight disengagement of ammonia was perceptible". Children analyzed by elemental analysis the residue left after repeated extractions with strong potassium hydroxide solution and found substantial quantities of nitrogen (11.05% and 9.54% in two analyses), giving the empirical formula $C_{11}H_{17}O_7N_2$. Children also found in the cantharides "a small portion of silica and magnesia, and a slight trace of manganese".

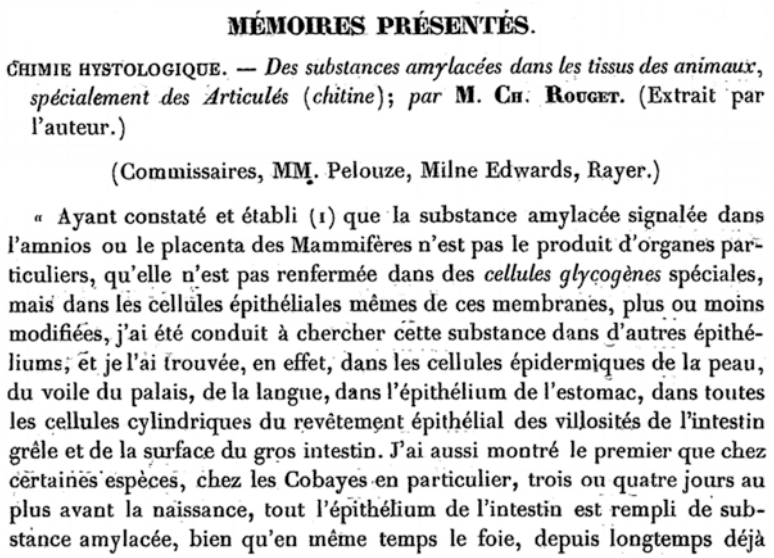
Children published his own observations in an appendix in the same journal (Children 1824). In this paper, he suggested that "Odier's test could have failed if the volatile acetic acid was evolved simultaneously with the ammonia". The resulting neutralization would give negative tests for ammonia and this had led Odier to conclude that nitrogen was absent. This suggestion appears to have been made in ignorance of Braconnot's work in which the evolution of acetic acid was reported. However, since the samples burnt by Odier had only been extracted with boiling water and not with hot caustic potash solutions, they would have contained a considerable amount of protein which should have given a positive test for nitrogen on burning. Hence, Children's explanation cannot be correct and the reason for Odier's negative result is unresolved (Roberts 1992). From the description of the process, it is probable that Children isolated chitosan rather than chitin (Roberts 1992).

1.2.5 Charles Rouget

The history of chitosan dates back to 1859 with the work of the French physiologist Rouget. Charles Marie Benjamin Rouget (1824–1904) was Professor of Physiology at the *Muséum d'Histoire Naturelle* in Paris (Gréhant 1904a, b). In 1859, Rouget found that boiling *chitine* in a concentrated potassium hydroxide solution under reflux rendered it soluble in dilute solutions of organic acids (Rouget 1859). This new product also gave a different color on treatment with an acidified iodine solution than did the original *chitine* (Fig. 1.6). Indeed, mixtures of iodine and zinc chloride gave blue or violaceous colors with *chitine*. Rouget was the first to describe the deacetylation of chitin, opening new possibilities for its use. He gave the name of the product "*chitine modifiée*", modified chitin. However, it was not until 1894 that Hoppe-Seyler named the tailored chitin, chitosan.

1.2.6 Georg Ledderhose

Georg Ledderhose (1855–1925) studied medicine in Strassburg as a pupil of Georg Albert Lücke (1829–1894). In 1875, Ledderhose, studying the hydrolysis of *chitine* with concentrated HCl, discovered *glykosamin* "*unter der gütigen leitung des herrn Hoppe-Seyler*", i.e. with the kind collaboration of Hoppe-Seyler, in Strassburg (Ledderhose 1876, 1878, 1880a, b). This was the topic of his doctoral thesis



MÉMOIRES PRÉSENTÉS.

CHIMIE HYSTOLOGIQUE. — *Des substances amylacées dans les tissus des animaux, spécialement des Articulés (chitine)*; *par* M. CH. ROUGET. (Extrait par l'auteur.)

(Commissaires, MM. Pelouze, Milne Edwards, Rayer.)

« Ayant constaté et établi (1) que la substance amylacée signalée dans l'amnios ou le placenta des Mammifères n'est pas le produit d'organes particuliers, qu'elle n'est pas renfermée dans des *cellules glycogènes* spéciales, mais dans les cellules épithéliales mêmes de ces membranes, plus ou moins modifiées, j'ai été conduit à chercher cette substance dans d'autres épithéliums; et je l'ai trouvée, en effet, dans les cellules épidermiques de la peau, du voile du palais, de la langue, dans l'épithélium de l'estomac, dans toutes les cellules cylindriques du revêtement épithélial des villosités de l'intestin grêle et de la surface du gros intestin. J'ai aussi montré le premier que chez certaines espèces, chez les Cobayes en particulier, trois ou quatre jours au plus avant la naissance, tout l'épithélium de l'intestin est rempli de substance amylacée, bien qu'en même temps le foie, depuis longtemps déjà

Fig. 1.6 First page of the memory of Rouget, presented at the *Académie des Sciences* Paris, where he described his *chitine modifiée* in 1859. (Rouget 1859)

presented in 1880 at the *Kaiser Wilhelms Universität*, Strassburg. Ledderhose subsequently worked as a surgeon at the Strassburg hospital and became Professor Extraordinary of Surgery in Strassburg in 1891. Later, he worked in Munich, where he became Honorary Professor.

In 1875, Ledderhose was working during the summer semester in the laboratory of his uncle, Friedrich Wöhler (1800–1882), at Göttingen (Foster and Webber 1961; Brimacombe and Webber 1964). One day, Wöhler had lobsters for lunch and, bringing back the shell to the laboratory, he gave it to his nephew. He told him to "find out what this was". The young Ledderhose treated lobsters with hot concentrated hydrochloric with the aim to identify the structure of the products. He found that the claws and the shells dissolved in this solution and that on cooling the solution yielded characteristic crystals. In collaboration with Hoppe-Seyler, Ledderhose identified the crystalline compound as a new nitrogen-containing sugar which he named *glykosamin*/*glycosamin*. In 1876, he published his first results in the journal *Berichte der Deutschen Chemischen Gesellschaft* (Ledderhose 1876) where he described the presence of nitrogen (6.49%) and proposed the formula described in Scheme 1.1. The new crystalline product differed from glucose in having an amine group in place of one hydroxyl. Later, Ledderhose also found that acetic acid was a product of hydrolysis of arthropod chitin (Ledderhose 1878, 1880). He estimated

$$COH.(CH.OH)_4.CH_2.NH_2 + HCl.$$

Scheme 1.1 *Glykosamin*: Structural formula proposed by Ledderhose (1876)

$$\underset{\text{chitin}}{C_{45}H_{26}N_2O_{10}} + 3H_2O = \underset{\textit{glycosamin}}{2C_6H_{13}NO_5} + \underset{\text{acetic acid}}{3C_2H_4O_2}$$

Scheme 1.2 Decomposition of chitin according to Ledderhose (1878)

the quantity of products and arrived at the chemical reaction described in Scheme 1.2, confirming the structural formula $C_6H_{13}NO_5$. Ledderhose noticed, together with the acetic acid, small quantities of other volatile fatty acids, especially formic and butyric. Similar results were previously published by Schmidt (1845) and Städeler (1859). However, Ledderhose did not prove that glucosamine and acetic acid were produced in equimolar amounts. Indeed, the stoichiometry of reaction was only determined in 1912 by Brach and von Fürth (1912).

Glykosamin/glycosamin was the first amino sugar isolated in 1876 by Ledderhose. The term glucosamine was nevertheless coined by Tiemann in 1884 (Tiemann 1884). Moreover, the presence of *glykosamin*/glucosamine as the repeated unit of chitin was finally confirmed a few years later by the works of Tiemann (Tiemann 1884; Tiemann and Landolt 1886), Schmiedeberg (1891), Winterstein (1893, 1894a, b, 1894c, 1895a, b, c, d), and Gilson (1894a, b, c, 1895a, b).

1.2.7 Felix Hoppe-Seyler

Felix Hoppe-Seyler (1825–1895) was a German physiologist and chemist. Ernst Felix Immanuel Hoppe, his name at birth, was also a pioneer of biochemistry and molecular biology (Baumann and Kossel 1895a; Noyer-Weidner and Schaffner 1995). In 1846, Hoppe enrolled as a medical student at Halle, where he began chemical laboratory work. A year later, he worked in the laboratory of Ernst Heinrich Weber (1795–1878) in Leipzig and studied chemistry, medicine and physiology with Otto Linné Erdmann (1804–1869) and with Karl Gotthelf Lehmann (1812–1863). Hoppe then completed his medical studies in Berlin, where he presented his doctoral dissertation in 1850 on the chemical and histological aspects of cartilage structure (Baumann and Kossel 1895a, b; Fruton 1990). In 1861, Hoppe-Seyler became full Professor of Applied Chemistry in the medical faculty of Tübingen and in 1872 full Professor in Physiological Chemistry and Hygiene in the medical faculty of the newly established German university in Strassburg.

In 1894, Hoppe-Seyler treated the shells of crabs, scorpions and spiders with potassium hydroxide at 180 °C and found a "new" product (Hoppe-Seyler 1894, 1895). Hoppe-Seyler, who did not refer to the *chitine modifiée* of Rouget, gave it the name of chitosan (Fig. 1.7) and pointed out different observations (Hoppe-Seyler 1894): i) this product was readily soluble in dilute acetic acid, in agreement with the observation by Rouget (1859), and in hydrochloric acid solution, ii) it could be precipitated from such solutions by addition of alkali; and iii) it began decomposing

610. F. Hoppe-Seyler. Ueber Chitin und Cellulose.

(Eingegangen am 4. December; mitgeth. in der Sitzung von Hrn. A. Pinner.)

In diesen Berichten 27, Heft 17, S. 3113—3115 ist von E. Winterstein soeben eine Mittheilung, betitelt »Ueber ein stickstoffhaltiges Spaltungsproduct der Pilzcellulose« veröffentlicht, die mir besonders durch ihren letzten Satz Veranlassung zu folgenden Bemerkungen giebt:

Unter Benutzung einer vor mehreren Jahren beschriebenen Isolirungsmethode, welche von Winterstein auch in der citirten Mittheilung erwähnt ist, habe ich mich seit längerer Zeit mit den der Cellulose verwandten Kohlehydraten von Thieren und Pflanzen beschäftigt und einige Resultate erhalten, deren kurze vorläufige Schilderung wohl jetzt zweckmässig sein wird, während die ausführlichen Mittheilungen erst später gegeben werden können.

Das Tunicin der Tunicaten hat sich auch bei der Behandlung mit Aetzkali bis 180° als übereinstimmend mit gewöhnlicher Cellulose erwiesen, dagegen hat das Chitin der Gliederthiere (untersucht wurden Panzer von Insecten, Krebsen, Scorpionen, Spinnen) eine recht merkwürdige Abweichung gezeigt. Während bei dem Erhitzen mit Aetzkali und ein wenig Wasser im Oelbade bis 180° (als Maximum) die Formen der Chitingewebe so wenig wie die Zellengewebe der Pflanzen bis hinab zu den Algen eine wesentliche Aenderung erkennen lassen, wird das Chitingewebe nach dieser Behandlung und sorgfältigem Auswaschen des Aetzkalis mit kaltem Wasser leicht löslich in verdünnter Essigsäure zur klaren Flüssigkeit gefunden und durch Alkalilauge wird aus dieser Lösung ein reichlicher voluminöser Niederschlag gefällt. Der Stickstoffgehalt des Chitins ist bei dieser Behandlung unverändert geblieben, aber im aufgelösten Aetzkali, mit dem das Chitin erhitzt war, fand sich Essigsäure und zwar so rein, dass nach Uebersättigen mit Schwefelsäure aus dem Destillate sofort das Baryumsalz und aus diesem das Silbersalz von berechneter Zusammensetzung erhalten wurden. Das Umwandlungsproduct des Chitins, welches neben Essigsäure entsteht, sich in Essigsäure, auch in äusserst verdünnter Salzsäure sehr leicht löst, dem ich den vorläufigen Namen Chitosan gegeben habe, zeigt insofern basische Eigenschaften, als es sich mit den Säuren leicht verbindet, beim Verdunsten der wässrigen Lösung der salzsauren Verbindung diese in quadratischen Krystallen liefert, die

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Fig. 1.7 First page of the article of Hoppe-Seyler published in 1894 in the journal Berichte where he introduced the name chitosan. (Hoppe-Seyler 1894)

at temperature 184 °C and was stated, surprisingly, to have the same nitrogen content as the original chitin. One year later his pupil Araki (1895) and later Löwy (1909) also reported similar conclusions.

Using Schmiedeberg's view of the constitution of chitin (Schmiedeberg 1891), Hoppe-Seyler clearly demonstrated the relationship between chitin and chitosan. When chitosan is treated with concentrated hydrochloric acid it, like chitin, yielded glucosamine (Hoppe-Seyler 1894, 1895). If heated with acetic anhydride, it yielded

a substance resembling chitin, which, when heated with potash at 180 °C, was resolved into chitosan and acetic acid.

At the same time, the product described by Hoppe-Seyler as a partially deacetylated, acid-soluble derivative of chitin was also prepared from fungal material by both Winterstein (1893, 1894a, b, 1895a, b, c, d) and Gilson (1894a, b, c). Hoppe-Seyler however claimed priority (Hoppe-Seyler 1894).

1.3 A Period of Confusion and Controversy: 1894–1930

From 1894 to 1930, chitin entered in a period of confusion and controversy. The names that marked this period most were Ernst Winterstein, Eugène Gilson, Sigmund Fränkel, Emil Fischer, James C. Irvine, Paul Karrer, and Albert Hofmann. During this period, research on chitin was mostly directed toward the study of its occurrence in living organisms, its determination and its chemistry. These works were even marred by frequently contradictory results and hot debate between the numerous and different laboratories, due mainly to confusion arising from the terminology of the different polysaccharides studied during the 19th Century, the lack of a systematic nomenclature, and also the lack of certainty concerning their structure (Haworth 1946; Bell 1949; Tracey 1957).

1.3.1 Nomenclature

Chitin first named *fongine* by Braconnot (1811) and then *chitine* by Odier (1823). However, other names have been proposed but have not gained acceptance and/or led extensive debate. These include elytrine by Children (1824), *entomaderme* by Lassaigne (1843a, b), *metacellulose* by Frémy (1855), fungus-cellulose, fungo-cellulose, fungal cellulose, or *pilzcellulose* by de Bary (1887) and Winterstein (1893), *entomeiline* by Packard (1886, 1898), pupine by Griffiths (1892a, b), and later mycetin by Ilkewitsch (1908). The American entomologists frequently used the term chitinous or chitinized in morphological or taxonomic descriptions (Ferris and Chamberlin 1928; Campbell 1929).

1.3.2 Ernst Winterstein

In August 1893, the Swiss chemist Ernst Winterstein (1865–1949), who removed fats and proteins from fungus, e.g. *Boletus edulis*, *Agaricus campestris*, and *Morchella esculenta*, found that the residue was insoluble in Schweitzer's reagent (Winterstein 1893). He concluded that it was a cellulose differing from that in tissues of higher plants and named it "fungus cellulose"/*pilzcellulose*. Winterstein also

reported that a nitrogenous substance and acetic acid were among the products of the acid hydrolysis of fungal chitin (Winterstein 1893). The 9 November of the next year, Gilson (1894a), in France, reported the presence of chitin in fungi, studied its chemistry and its conversion to *mycosine*/mycosin (chitosan). He also noted the presence of glucosamine, a "new nitrogenous substance". Six days later, Winterstein published another paper dealing with "fungus cellulose", the nitrogen-containing material obtained from the same fungi by fusion with caustic potash solution at 180 °C (Winterstein 1894a). In this work, Winterstein also confirmed identification of glucosamine in the products when heated with hydrochloric acid, the same glucosamine previously described by Ledderhose. The next year, Winterstein showed that mycosin from "fungus cellulose" was decomposed in acid solution into D-glucose, other hexoses and then into acetic acid and an undetermined nitrogenous organic substance (Winterstein 1895c, 1895d). When heated with concentrated hydrochloric acid, it yielded a crystallisable fission product, which proved identical with the hydrochloride of chitosamine, $C_6H_{11}O_5NH_2$, HCl, at that time erroneously termed glucosamine. The same behavior was exhibited by *chitine*, the substance discovered by Odier. As also shown by Ledderhose in 1876, this substance furnished under similar treatment the glucosamine, and that too in the state of hydrochloride. Discussion of whether fungal cellulose was identical with the cellulose of higher plants had been vigorous, the evidence adduced being based on solubility behavior and staining reactions summarized by Winterstein (1894b). The use of name cellulose with reference to fungal chitin as proposed by Winterstein continued for several years.

1.3.3 Eugène Gilson

The chemical similarity of fungal and animals chitins is attributed to Gilson. Eugène Gilson (1862–1908), a pupil of Hoppe-Seyler, was Professor at the University of Gand (Leboucq 1913). Gilson believed that fungus tissue did not contain cellulose. In 1893, Gilson was unable to obtain crystalline cellulose from *Mucor vulgaris*, *Thamnidium vulgare*, and *Agaricus campestris*, while he succeeded easily with plant tissue (Gilson 1893). One year later, Gilson studied the presence of chitin in fungi (Gilson 1894a, b, c) and noted that its elemental composition was in close agreement with previously reported analyses for chitin of insect origin. Gilson also noted that the residue obtained after treating certain fungi with dilute sulphuric acid, and then dilute sodium hydroxide under reflux gave glucosamine on hydrolysis with hydrochloric acid and that, just as in the case of chitin, acetic acid was produced during hydrolysis.

By fusing cell preparations, e.g. *Agaricus campestris*, ergot of rye *Secale cornutum*, with caustic potash at 180 °C, Gilson (1894a, b, c) also obtained a residue (in sulphate or chlorhydrate form), not of cellulose but a substance insoluble in Schweitzer' reagent, and to which he gave the name of *mycosine*/mycosin with the formulate $C^{14}H^{28}Az^2O^{10}$. One year later, Araki (1895), studying the formation of

chitosan/mycosin from chitin, proposed the composition $C^{18}H^{30}N^{2}O^{10}$ for chitosan. Gilson showed that *mycosine* was soluble in 2 to 3 per cent hydrochloric acid or in very dilute acetic acid (Gilson 1895). A solution of iodine in potassium iodide, containing a trace of free acid, gave a reddish violet stain. Zinc-iodo-chloride solution varied in action in accordance with the amount of zinc chloride present, 50% producing a blue to blue-violet coloration. These reactions closely resembled those of cellulose. Gilson was also among the early researchers to point out that chitin may be associated with other carbohydrate materials and substances analogous or identical to those found in phanerogams. The name *mycosine*/mycosin, like the alternative names for chitin, has never come into general usage.

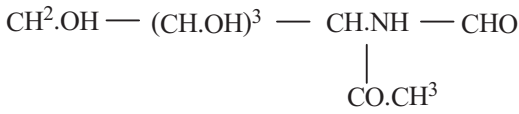
1.3.4 Sigmund Fränkel

Sigmund Fränkel (1868–1939) was a Polish-born physiological chemist and pharmacist who worked in Vienna. Fränkel studied medicine at the Universities of Prague, Freiburg, and Vienna. He played an important role in research into chitin chemistry (Fränkel 1898; Fränkel and Jellinek 1927; Fränkel and Kelly 1901a, b, 1903). In 1901, using a milder acid hydrolysis of purified chitin (cold concentrated H_2SO_4 for two days, room temperature), Fränkel and his colleague, Agnes Kelly, isolated five fractions by precipitation with alcohol followed by ether; the last and most soluble fraction was shown to be N-acetylglucosamine (Fränkel and Kelly 1901a, b). They demonstrated that reaction yielded not only glucosamine/chitosamine, formula $C_6H_{13}NO_5$, and acetic acid but also small amounts of monoacetylglucosamine, formula $C_6H_{15}NO_6$ (Scheme 1.3). This compound was identical with this previously obtained by N-acetylation of D-glucosamine by Breuer (1898). Fränkel and Kelly (1903) concluded that chitin was an acetylated and aminated polysaccharide. A detailed discussion on the hydrolysis of chitin and on the structure of products can be found in the memoir by Bounoure (1919).

1.3.5 Emil Fischer

Emil Fischer (1852–1919, Nobel Prize in 1902) was a German organic chemist and also a pioneering figure in biochemistry (Freudenberg 1967; Lichtenthaler 2002). While working at the University of Munich in the early 1880s, Fischer found that phenylhydrazine converted sugars into osazones whose crystals had characteristic forms that could be identified. The chemistry of phenylhydrazine

Scheme 1.3 Structure of the acetylglucosamine proposed by Fränkel and Kelly (1901a)



was accidentally discovered by him in 1884 when he worked in Strassburg (Fischer 1884). Fisher also proposed the synthesis of glucosamine and he has written upon the value of this discovery: "The synthesis of glucosamine showed it to be an intermediate grape sugar and the α-amino acids, so providing one of the longest sought-for bridges between the carbohydrates and the proteins" (Fischer 1912; Bunge 1912).

At the beginning of 1900s, Fischer and its Ph.D. student Hermann Leuchs proposed the synthesis of glucosamine and established its constitution (Fischer and Leuchs 1902, 1903). By the combination of D-arabinose and ammonium cyanide, or D-arabinose-imine with hydrogen cyanide, D-glucosaminic acid was obtained and its lactone reduced to glucosamine. Glucosamine formed a penta-acetyl derivative and also an oxime, semi-carbazone and phenyl hydrazine. However, it could not be converted into glucose, though it gave glucose phenyl osazone when heated with phenyl hydrazine (Fischer and Leuchs 1903). Nitrous acid converted it into a new compound regarded as a sugar, and also termed chitose ($C_6H_{10}O_5$). This formed chitonic acid when oxidized. Glucosamine was then regarded as a derivative of chitose, and termed chitosamine, confirming the conclusions previously reported by Fränkel and Kelly (1901a, b). In another contribution, chitose was shown by Fischer and Andreae (1903) to be a hydrated furfurane derivative rather than a true sugar, formed by simultaneous elimination of the amino group and anhydride formation.

1.3.6 James Irvine

James Colquhoun Irvine (1877–1952) was a Scottish organic chemist, Professor of Chemistry at the University of St Andrews. Irvine studied chemistry at the University of Leipzig under the supervision of Friedrich W. Ostwalt (1853–1932, Nobel Prize in chemistry in 1909). Irvine was appointed Professor of Chemistry in 1909 at the University of St Andrews. Irvine (1909) first reported the specific rotatory power of chitin in hydrochloric acid solution ($^{20}[\alpha]_D$ – 14.1°) and its index of refraction (1.525). He also studied the action of acetyl-bromide on glucosamine hydrochloride with the object to clarify the constitution of structural unit of chitin. Although the theoretical amount of nitrogen was evolved when glucosamine was decomposed by nitrous acid, the product of the change was not a simple hexose, but the hydrated furan derivative known as chitose. Chitose was formed under all conditions when nitrous acid acted on glucosamine, and this accounted for the alternative name chitosamine (Irvine 1909; Irvine et al. 1911). Pursuing his work on chitin derivatization into aceto-halogen derivatives, Irvine with his pupil Alexander Hynd concluded that glucosamine may be derived from either glucose (Irvine and Hynd 1912) or mannose (Irvine and Hynd 1914) according to the method of preparation. Glucosamine as an α-amino-derivative had the D-glucose configuration. Their conclusions were in agreement with those published by Fischer and Leuchs (1903) and by Hamlin (1911). However, Irvine and Hynd stated that no rigorous proof could be offered in support of this latter assumption and "the

displacement of any group by any other group may be accompanied by a Walden inversion". The stereochemical arrangement of the amino-group was thus uncertain.

Chitosamine, 2-amino-2-deoxy-D-glucose, was then considered as an amino-hexose from chitin, and alternatively described as glucosamine or mannosamine. However, there was a period of confusion for the nomenclature related to this designation until the work of Karrer.

1.3.7 Paul Karrer

In the early 1920s, the Swiss chemist Paul Karrer (1889–1971), Nobel Prize winner in 1937 for his work on vitamins (the prize was shared with British chemist Norman Haworth), published several studies on the chemistry and biochemistry of chitin (Karrer and Smirnoff 1922; Karrer and Hofmann 1929; Karrer and von François 1929; Karrer et al. 1924; Karrer 1930; Karrer and White 1930). Karrer studied chemistry at the University of Zurich with Alfred Werner (Nobel Prize in chemistry in 1913) and obtained his thesis in 1911. After a position of researcher at the Georg-Speyer Haus Foundation in Frankfurt working with Paul Ehrlich (Nobel Prize in medicine in 1908), Karrer in 1919 accepted the position of Professor of Organic Chemistry at the *Chemisches Institut* of Zürich and succeeded Werner as director of this institute (Dahn et al. 1969; Eugster 1972; Roche 1972; Wettstein 1972; Beer 1977). Karrer started studies on the chemistry of sugars and polysaccharides such as starches, dextrins, glycogen, inulin, cellulose, chitin, etc.

Karrer studied the degradation, chemistry and biochemistry of chitin and he also prepared different derivatives such as glucosamine or chitosamine and N-acetylglucosamine (Karrer and Smirnoff 1922; Karrer et al. 1924). The powerful acid hydrolysis of chitin led to the removal of the acetyl group from the macromolecule, giving thereby both glucosamine and acetic acid, confirming previous papers on this topic. Karrer demonstrated that complete acid hydrolysis of chitin yielded D-glucosamine and acetic acid in nearly theoretical amounts if it was assumed that polysaccharide was composed only of mono-acetyl-D-glucosamine units (Karrer and Smirnoff 1922; Karrer et al. 1924), in agreement with the result of Brach and von Fürth (1912). Later, Zechmeister and Tóth (1931), and Bierry et al. (1939), using chemical procedures and enzymatic reactions, reported a similar conclusion.

In 1929, Karrer and his student Hofmann reported the formation of N-acetylglucosamine (in 50% yield) by the action of the gut contents of the edible snail *Helix pomatia* on lobster chitin (Karrer and Hofmann 1929). The same year, Karrer and another pupil, Götz von François, reported the isolation of 80% of the theoretical amount of acetylglucosamine from an enzymatic digest of a fungal chitin from *Boletus edulis* (Karrer and von François 1929). Karrer's group also showed that re-acetylated chitosan was hydrolyzed by chitinases with the produc-

tion of acetylglucosamine. Chitosan re-acetylated remained a substrate for *Helix* chitinase while chitosan derivatives containing formyl, propionyl, butyryl and benzoyl groups were not substrates (Karrer and von François 1929; Karrer 1930; Karrer and White 1930).

Karrer (1930) also studied the preparation of chitosan. He pointed out that chitosan gave low nitrogen values in a Kjeldahl analysis due to the hydrolysis of some of the amine groups to hydroxyls during the synthesis of chitosan. Later, this was confirmed by Clark and Smith (1936). For Karrer, chitosan was a mono-acetyl-diglucosamine and this was demonstrated by the fact that chitinase acting on chitosan gave both N-acetylglucosamine and glucosamine (Karrer 1930). Finally, Karrer prepared several derivatives of chitin. For instance, he suggested that phosphoric esters might be of interest in the metabolism of chitin (Karrer et al. 1943). He also studied their anticoagulant activity.

1.3.8 Albert Hofmann

Albert Hofmann (1906–2008) began in 1925 his studies in chemistry at the *Chemisches Institut* and prepared his doctorate under the supervision of Karrer on the elucidation of the sugar building blocks of chitin (Hofmann 1929; Finney and Siegel 2008; Hagenbach et al. 2013). Hofmann presented his dissertation entitled "*Über den enzymatischen abbau des chitins und chitosans*" in the spring of 1929. The same year, after leaving university, Hofmann took a job with Sandoz Laboratories in Basel, where he stayed for more the four decades. His main interest was the chemistry of plants and animals. Hofmann also conducted important research on the chemical structure of chitin.

Hofmann's thesis described the first enzyme that very efficiently degraded chitin (Hofmann 1929; Karrer and Hofmann 1929; Finney and Siegel 2008), obtained from the crude extracts of a common snail, *Helix pomatia*. Hofmann and Karrer (1929) proposed the name chitinase for the active principle of the gastro-intestinal juice of the vineyard snail used. Its action on partially deacetylated chitin (chitosan) led to the formation of oligosaccharides, resistant to acid hydrolysis, containing N-acetyl groups and forming insoluble sulphates. In 1935, Freudenberg and Eichel have reported that the same chitinase destroyed the blood activity of human urinary mucosubstances and liberated N-acetyl-aminosugar from them (Freudenberg and Eichel 1935). Hofmann and Karrer (1929) conclusively demonstrated that chitin was a polymer of N-acetylglucosamine, and chitosan a polymer containing both glucosamine and N-acetylglucosamine. However, it was only in 1946 that Purchase and Braun (1946) clearly elucidated the chemical structure of chitin using hydrolyzing experiments, and in 1977, Muzzarelli reported its distribution, along with that of its derivative chitosan, in the living species (Muzzarelli 1977).

1.4 Exploration: 1930–1950

At the end of the 1920s, the first X-ray diffraction patterns showed considerable similarity to patterns from cellulose and strengthened but did not prove the case in favor of glucosamine, the structural unit of chitin (Herzog 1924; Gonell 1926; Meyer and Mark 1928). Glucosamine was then the object of numerous fundamental studies (Karrer and Smirnoff 1922; Fränkel and Jellinek 1927; Bergmann and Zervas 1931; Elson and Morgan 1933; Bergmann et al. 1934; Kawabe 1934; Morgan and Elson 1934; Cox et al. 1935; Cutler et al. 1937; Cox and Jeffrey 1939; Cutler and Peat 1939; Bierry et al. 1939; Freudenberg et al. 1942). However, much controversy and debate surrounded the constitution of glucosamine, and in particular the stereochemical position of the amino group (Meyer 1942). Many reactions indicated that the amino group had the same position as the hydroxyl group in mannose, while other reactions suggested a relationship with glucose.

Between 1930 and 1950, X-ray analysis became the most reliable method for the differentiation between chitin and cellulose in cell walls of fungi (Farr and Sisson 1934; Clark 1934; Mark 1943; Frey 1950). During this period, chitin and chitosan attracts considerable attention with the exploration of natural fibers. In the mid-1930s, the first chitosan films and fibers were patented by George W. Rigby (Rigby 1936a, b, c, d, e, 1937). There was also the first use in papermaking industry (Lubs 1937; Larson 1939, 1940), textile (Heckert 1937; Arnold 1939), photography (Marasco 1938, 1939; White 1944), and as adhesives (Maxwell 1939). The names that marked this period were Kurt H. Meyer, Max Bergmann, László Zechmeister, Norman Haworth, and Albert G. Richards.

1.4.1 Kurt H. Meyer

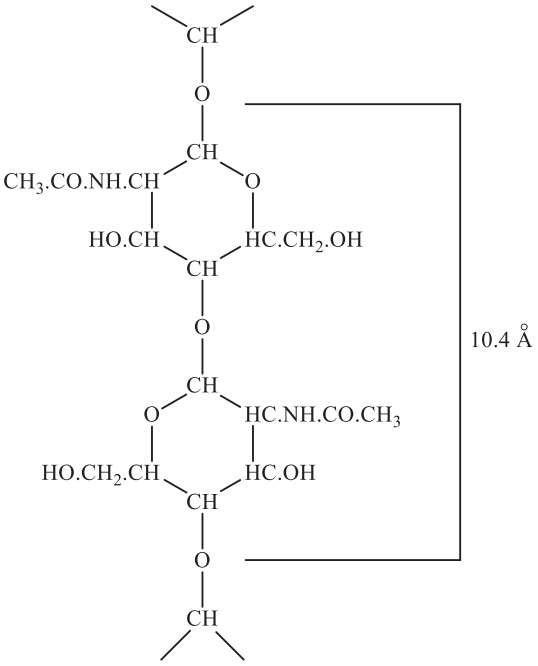
Kurt Heinrich Meyer (1883–1952) was a German chemist of Baltic origin (born in Dorpat). Meyer studied chemistry in Leipzig. After obtaining his Ph.D., he traveled with his father (a famous pharmacologist) through several continents, and spent a year in Rutherford's laboratory in England. After his return to Germany, Meyer went to Bayer's laboratory. After the war, he returned to Munich as Professor in Chemistry until 1920, when he became head of a research laboratory of the I.G. Farben Company. In 1932, Meyer accepted a position at the University of Geneva and became Director of the Organic Chemical Institute (Mark 1952; Jeanloz 1956). Meyer published several important contributions on natural and synthetic polymers and their chemistry. Many of them were devoted to chitin (Meyer and Mark 1928; Meyer and Pankow 1935; Meyer and Wehrli 1937; Meyer 1942, 1950).

In 1924, Herzog was the first to show the crystalline nature of chitin using X-ray diffraction Herzog (1924). The crystalline nature was confirmed and amplified 2 years later by Gonell, under the supervision of Herzog, who arrived at a hexagonal unit cell (Gonell 1926). Indeed, Gonell, studying X-ray data of chitin (*Goliathus*

giganteus), proposed and discussed a rhombic cell with the dimensions a = 19.42 Å, b = 10.40 Å, and c = 11.58 Å (the cell contained 10 acetylglucosamine units). However, Gonell eliminated it because such a cell could not contain the ten sugar units which were indicated by density measurements (Gonell 1926). He preferred a hexagonal unit cell with 18 glucosamine units. His work was comprehensively discussed by Professor Meyer.

Meyer and Mark (1928), aware of the biological analogy between chitin and cellulose and of the similarity of their structure, proposed a rhombic unit cell. The analogy between the two polysaccharides was apparent in the main features of the X-ray diagram. This analogy led Meyer and Mark to the conclusion that the units of glucosamine were united in the same manner as the glucose units in cellulose, that was, in 1,4-β linkage, each chain having a diagonal screw axis. The authors ascribed to chitin the constitution shown in Scheme 1.4 and assumed that chitin had a micellar structure of parallel oriented chains similar to that cellulose. At the beginning of the 1930s, this structure received important support when Bergman isolated chitobiose, a product of acetolysis (Bergmann et al. 1931a, b, c). The assumption that chitin had a micellar structure of parallel oriented chains similar to that of cellulose was also confirmed in the same period by the demonstration of presence of β-glucosidic linkages by Zechmeister using incomplete acidic hydrolysis (Zechmeister et al. 1932; Zechmeister and Tóth 1933). However, in the mid-1930s, Schorigin stated that the unit of the structure was neither glucose nor mannose, but another sugar (named chitose), differing in configuration (Schorigin and Hait 1934, 1935; Schorigin and Makarowa-Semljanskaja 1935a, b).

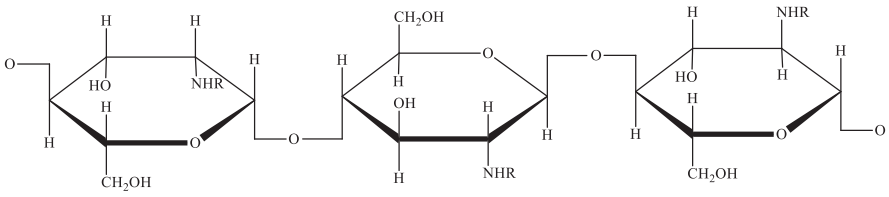
Scheme 1.4 Structural formula of chitin proposed by Meyer and Mark (1928) in agreement with that suggested by Löwy (1909)



A detailed analysis by Meyer and Pankow (1935) provided the basis for the structure commonly accepted in the 1950s (Meyer 1950; Whistler and Smart 1953). This analysis was made on a tendon of the rock lobster *Palinurus vulgaris*, and led to a rhombic cell comprising four chitobiose units with the dimensions a = 9.40 Å, b = 10.46 Å (fiber axis), and c = 19.25 Å. Indeed, the cell contained 8 acetylglucosamine units (7.9 calculated from the density = 1.415). The analogy with cellulose was apparent in the main features of the diagram proposed for the unit cell of chitin: equal numbers of chains were arranged in opposing directions, the rings followed one another in a diagonal screw sequence and all rings lay flat in one plane. These results have been confirmed by Clark and Smith (1936) but stated the dimension of the a axis as 9.25 Å. Later, Lotmar and Picken (1950) using chitin from the same source, i.e. *Palinurus vulgaris*, confirmed the a and c values but preferred 10.27 Å for b. Similar figures were also obtained using fungal and insect chitin. Heyn (1936a, b) stated dimensions of a = 9.70, b = 10.4, c = 4.6 Å for fungal chitin and the diagrams obtained were almost the same as those from control chitin from cockroaches. Similar X-ray patterns were obtained from chitin in plant cell walls (Khouvine 1932; Heyn 1936c; van Iterson et al. 1936).

Meyer and Pankow (1935) also indicated that acetyl groups alternated from one side of the chain to the other on passing from one residue to the next (evidence in favor of β-linking) and that while chains along the a axis ran in the same direction, the direction was reversed in their neighbors in the direction of the c axis. This alternation in the direction of contiguous chains was also found in cellulose (Meyer and Wehrli 1937; Meyer 1942). Later, the crystal structure determined by Meyer and Pankow (1935) was slightly modified by Darmon and Rudall (1950), who also suggested that there were C=O---H-N and C=O---H-O hydrogen bonds as well as free O-H and N-H groups in the crystal. Pursuing his studies on chitin chemistry, Meyer reported that solutions of fungal chitin in acids had the same viscosity as those of animal chitin (Meyer and Wehrli 1937). It was noted that the viscosity of solutions of chitin in nitric acid solution was of the same order as that of similar solutions of cellulose indicating a degree of polymerization of more than a hundred. Fikentscher (1932) previously reported similar conclusion.

At the beginning of the 1940s, it was clear from all the results published that animal and plant chitins were essentially similar and that, as was suggested by Meyer, chitin closely resembled cellulose in structure and both consisted of long primary valence chains of glucose residues (Picken 1940; Meyer 1942). The constitution of chitin shown in Scheme 1.5 has been recognized. Later, this structure was



Scheme 1.5 Structural formula of chitin accepted in the 1940s

also identified by enzymatic analysis and infra-red spectroscopy (Darmon and Rudall 1950; Orr 1954; Brock 1957; Spedding 1964).

1.4.2 Max Bergmann

At the end of the 1920s, there seemed little doubt that nearly all of a purified chitin can be regarded as a polymeric form of N-acetyl-2-amino-2-deoxy-D-glucose (Tracey 1957; Foster and Webber 1961; Kent 1964). However, it remained to determine the mode of linkage. On this topic, Bergmann published several papers (Bergmann and Zervas 1931; Bergmann et al. 1931a, b, c). Bergman was also the first to suggest that glucosamine had the typical pyranose ring structure (Bergman et al. 1934). In the proposed structure, the six atoms of the ring were nearly coplanar, the oxygen atom being slightly displaced out of the plane of the carbon atoms.

Max Bergmann (1886–1944), a German biochemist, received his Ph.D. in 1911 and became the assistant to Emil Fischer at the University of Berlin. After Fischer's death in 1919, Bergmann automatically became his scientific executor, assuming responsibility for the completion and publication of unfinished researches (Helferich 1969; Katsoyannis 1973; Lichtenthaler 2002). In 1920, he habilitated at the University of Berlin and was appointed to head the newly established *Kaiser-Wilhem Institut für Lederforschung* in Dresden. In 1933, he left Germany for the United States and he accepted a position of researcher at Rockefeller University in New York City. Bergmann is considered a pioneer of applied sciences and of molecular biology.

With his pupil Leonidas Zervas (Katsoyannis 1973), Bergmann in 1931 was the first to isolate a crystalline disaccharide, as the octa-acetate, by acetolysis of chitin (Bergmann et al. 1931a). He assigned it the name chitobiose. The reducing disaccharide chitobiose was the first low molecular weight polymers obtained from chitin. The product contained two N-acetyl and six O-acetyl groups. Further acetylation confirmed the presence of six hydroxyl groups. Its configuration was identical with the cellobiose molecule except for the substitution of acetylamine groups for the hydroxyl group on carbon-2. The reactions of chitobiose octa-acetate (i.e. oxidation with iodine in alkaline conditions followed by hydrolysis) led to the conclusion that a 1,4-linkage was likely and X-ray evidence and changes of rotation ($^{20}[\alpha]_D$ -14° → + 56°) on hydrolysis indicated a β-form, in agreement with the results published by Meyer and Mark (1928) and by Karrer (Karrer and Hofmann 1929; Karrer and von François 1929). All the results clearly demonstrated that carbon-1 as well as carbon-2 of the reducing moiety cannot be involved in the glycosidic linkage. Shortly thereafter, the postulated β-glycosidic linkage was also confirmed by Zechmeister (Zechmeister et al. 1932; Zechmeister and Tóth 1933).

1.4.3 László Zechmeister

László Zechmeister (1890–1972) was born in the city of Györ, in Hungary. In 1907, he commenced his studies in chemistry at the *Eidgenössische Technische Hochschule* in Zurich, under the guidance of Richard Willstätter (1872–1942, Nobel Prize in chemistry in 1927). Zechmeister received his degree as technical chemist on 1911 and then worked at the Kaiser Wilhelm Institute for Chemistry in Berlin-Dahlem from 1912 to 1914. During this period, Zechmeister received his doctorate on "*Zur kenntnis der cellulose und des lignins*". After the World War I, in 1921, Zechmeister accepted a position as instructor at the Royal Danish Agriculture and Veterinary Academy in Copenhagen until 1923. Then, he became Professor of Medical Chemistry at the University of Pécs, Hungary (he was only 33 years old). Zechmeister is considered as a pioneer of chromatography (Ettre 1989; Wirth 2013). He published a series of publications on chitin chemistry (Zechmeister and Tóth 1931, 1932, 1933, 1934, 1939a, b, c; Tóth and Zechmeister 1939; Zechmeister et al. 1932, 1939a, b).

In 1931, chitobiose, disaccharide found by Bergmann (Bergmann et al. 1931a) was also isolated by Zechmeister and his colleague Géza Tóth from the products of partial acid hydrolysis of chitin (Zechmeister and Tóth 1931). Chitobiose, originally isolated from lobster shells, was isolated from beetles, snail radulae, and also fungi (Zechmeister and Tóth 1934; Tóth and Zechmeister 1939). In addition to chitobiose, partial degradation of chitin also yielded a trisaccharide chitotriose from the acetolysis mixture, which had been characterized as its crystalline acetate, chitotriose undeca-acetate (Zechmeister and Tóth 1932). A more detailed account about the D-glucosamine or chitosamine was then provided. The authors, using X-ray diffraction measurements and rotary change experiments (Zechmeister and Tóth 1932, 1933), also suggested the β-linkage, in agreement with studies published by Karrer (Karrer and Hofmann 1929; Karrer and von François 1929) and Bergmann et al. (1931a). Zechmeister and Tóth (1934) comprehensively discussed the occurrence of chitin in plants and animals, its characteristics and analysis. A few years later, using enzymic studies, Zechmeister also confirmed the β-linkage (Zechmeister and Tóth 1939a, b, c; Zechmeister et al. 1939a, b).

1.4.4 Norman Haworth

Walter Norman Haworth (1883–1950) was a British chemist at the University of Birmingham, known for his work on ascorbic acid and the development of the Haworth projection used in organic chemistry to characterize sugar structures. He studied organic chemistry at the University of Göttingen earning his Ph.D. in Otto Wallach's laboratory. Haworth received the Nobel Prize in chemistry in 1937, shared with Karrer, for his work on carbohydrates and vitamin C.

In 1939, Haworth, Lake and Peat proposed a synthetic method which distinguished between the alternative configurations, glucose or mannose, for glucosamine (chitosamine). The fission by alkali such as sodium methoxide of the ethylene oxide ring in anhydro-sugars leads to the formation of two isomeric sugars, consequent upon the independent rupture of the two bonds of the oxide oxygen atom. In each case, ring opening was accompanied by a Walden inversion at the carbon of the epoxide ring at which the attack occurred and to which the amine group therefore became attached. The method has been to prepare a dimethyl 2,3-anhydro-methylmannoside which has been shown to give rise, with sodium methoxide or sodium hydroxide, to both a glucose derivative and an altrose derivative. The opening of the anhydro-ring in the dimethyl 2,3-anhydro-methylmannoside was then carried out by the agency of ammonia, which gave rise to a derivative of 3-amino-altrose on the one hand and of 2-amino-glucose on the other. The latter was shown to be identical with chitosamine, which might be considered configurationally to be glucosamine and therefore related to the parent sugar glucose (Haworth et al. 1939). It should be pointed out that two other groups came to similar conclusions the same year (Neuberger and Pitt Rivers 1939; Cox and Jeffrey 1939).

1.4.5 Albert Glenn Richards

At the end of the 1940s, the works of Albert Glenn Richards, an American Professor of Zoology at the University of Minnesota, on the biochemistry of chitin were acknowledged to have made an important contribution (Richards 1947a, b, 1949, 1951, 1952, 1958; Richards and Anderson 1942; Richards and Cutkomp 1946; Richards and Korda 1948; Richards and Pipa 1958). From 1890 to 1930, the most extensive work on chitin in fungi has been done with the genera of *Aspergillus*, *Agaricus*, *Boletus*, and *Phycomyces*. The first and complete list of forms tested in the fungi was only reported in 1951 by Richards in his book on the integument of the arthropods, which really set the benchmark for chitin zoological research (Richards 1951). This monograph is considered as a fundamental guide to the voluminous literature on the subject. Richards also reviewed some histochemical methods for the detection of chitin. Another fundamental book on the biology of the arthropod cuticle was published in 1975 by Anthony C. Neville (Neville 1975).

1.5 The Period of Doubt: 1950–1970

1.5.1 Literature Review

During the period of exploration (1930–1950), chitin and chitosan attracted considerable attention with the exploration of natural fibers and the first applications but lack of adequate manufacturing facilities and mostly cutthroat competition from synthetic polymers restricted their commercial development. From 1950 to 1970, chitin and chitosan entered in a period of doubt although much progress has also been made on their isolation, production and fundamentals (Wigglesworth 1948, 1957; Meyer 1950).

The first books on chitin were the well-known monographs on the integument of the arthropods by Richards edited in 1951 (Richards 1951) and on the biochemistry of aminosugars edited in 1955 by Paul Welberry Kent and Michael Wellesley Whitehouse (Kent and Whitehouse 1955). A few years later, other comprehensive books were published by Peter Bernfeld on the biogenesis of chitin (Bernfeld 1963), by Elwyn T. Reese on a discussion of the advances in the enzymic hydrolysis of natural substances (Reese 1963), and by Charles Jeuniaux on chitin and its enzymatic breakdown (Jeuniaux 1963).

In 1953, Roy Lester Whistler and Charles Louis Smart wrote a short chapter on chitin chemistry in their famous book "Polysaccharide Chemistry" (Whistler and Smart 1953). In 1955, M.V. Tracey published a review on the detection and determination of chitin in plant materials, and the quantitative analytical methods (Tracey 1955). Two years later, Tracey also published a detailed review entitled "chitin" (Tracey 1957). In 1958, Allan B. Foster and Maurice Stacey published a detailed chapter on the aminosugars and chitin in Encyclopedia of Plant Physiology (Foster and Stacey 1958). Biological aspects of chitin were summarized in general reviews by Richards (1958), Picken (1960), Jeuniaux (1963), and K.M. Rudall (1963), chemical aspects by Foster and Webber (1961), and its production by James N. BeMiller (BeMiller 1965). Later, Rudall also addressed the concept of the chitin-protein. He detailed the chitin-protein complexes and their conformation in two comprehensive reviews (Rudall 1967, 1969).

In 1964, Paul W. Kent published a comprehensive review on chitin and mucosubstances (Kent 1964). The same year, John S. Brimacombe and J.M. Webber discussed the chemical structure, distribution and isolation of mucopolysaccharides (Brimacombe and Webber 1964). The progress on chitin orientation in cuticle and its control were summarized by Neville (1967). Three chapters by Friedman (1970), Honke and Scheer (1970), and Jeuniaux (1971) were devoted to the zoological importance of chitin and its role in biochemical evolution. The first interdisciplinary book on chitin was published in 1973 (Muzzarelli 1973).

1.5.2 Selected Highlights

From this period of doubt (1950–1970), I choose to highlight the following studies. Until the beginning of the 1950s, the methods for isolating pure chitin by decalcification with acids and deproteinization with alkalis proposed in the nineteenth century by Odier, Children, Gilson and Winterstein, and later by other colleagues such as Scholl have continued virtually unchanged (Kent 1964). Between 1950 and 1960, "new" methods for the isolation of chitin were proposed by Blumberg (Blumberg et al. 1951), Hackman (Hackman 1953a, b, 1954, 1959, 1960, 1962; Foster and Hackman 1957), Roseman (Horowitz et al. 1957; Blumenthal and Roseman 1957), BeMiller (1965), and Broussignac (1968). Methods for the isolation of a series of oligosaccharides (Barker et al. 1957, 1958) and for the production of chitosan (Wolfrom 1958; Wolfrom et al. 1958; Wolfrom and Han 1959; Horowitz et al. 1957; Broussignac 1968) from chitin have also been proposed. All these methods were listed by Muzzarelli (1973).

In 1950, Jeanloz, studying periodate oxidation of chitin and chitosan, demonstrated that only 1-4-linkages were present in these polysaccharides (Jeanloz 1950; Jeanloz and Forchielli 1950, 1951). The same year, Darmon and Rudall (1950), studying the infra-red spectra of chitin, were able to identify the principal bands associated with the amido and hydroxyl groups vibrations and observed their dichroism. The authors correlated the infra-red absorption spectrum with the X-ray data in order to give a detailed structure for chitin. Darmon and Rudall (1950) also studied the infra-red spectra of deacetylated chitin and chitin nitrate.

In 1958, Giles and co-workers reported that chitin was a polymer of the amino sugar acetylglucosamine (Giles et al. 1958). However, in a small percentage of residues the acetyl group may be missing, leaving glucosamine, so that the chain as a whole may be positively charged. This could be important in chitin-protein cross-linking (Hackman 1960) and as one of the factors involved in orientation of the chitin chains (Attwood and Zola 1967; Neville 1967). At the beginning of the 1960s, it is evident that chitin, considered exclusively as a homopolysaccharide, was not normally found, and *in situ* it was associated with other substances, notably proteins, by hydrogen bonds and covalent linkages (Whistler and Smart 1953; Rudall 1963, 1965; Kent 1964). A convenient method of distinction proposed by Hackman (1960) reserved the name chitin for the chemically purified material and native chitin for the complex in which it is involved in tissues. The chemical structure of chitin was agreed to be that of a long unbranched polysaccharide in which N-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucopyranose) residues were linked in the β-(1 → 4) positions. At the end of the 1960s, it was accepted that three different molecular systems occurred as the chief skeletal support of living organisms, namely the cellulose systems present in plants, the collagenous systems present in animals, and the chitinous system (Rudall 1965, 1967, 1969; Rudall and Kenchington 1973). Chitin occurred as an alternative to cellulose in plants and as an alternative to collagen in animals. Significant progress was made in the chemistry and the production of chitin (Brimacombe and Webber 1964; Conrad 1966).

Although initiated in 1920s, an important contribution was made on the structure of "purified" chitin and its polymorphism during the period 1950–1970. Indeed this was the subject of hot debate between the different laboratories (Kreger 1954; Rudall 1955). X-ray fiber diagrams of oriented chitin samples finally showed an obvious similarity to those of cellulose. Comparison of the X-ray data for chitin from different sources had also revealed the existence in nature of more than one polymorphic form, namely α-chitin (Meyer and Mark 1928), β-chitin (Lotmar and Picken 1950) and γ-chitin (Rudall 1963). The structure of α-chitin has been investigated more extensively than that of either the β- or γ-form because it was the more common polymorphic form. The three forms have been found in different parts of the same organisms, suggesting that these forms were relevant to the different functions and not to animal grouping. However, it was difficult to assign a physiological role to these crystalline forms.

As already mentioned, the earliest X-ray investigation was that of Gonell (1926) whose results formed the basis for the discussion of Meyer and Mark (1928) on α-chitin. In this paper, a structure analogous to that of cellulose was first proposed. They established the unit cell as orthorhombic. The crystal structure of α-chitin was confirmed by Meyer and Pankow (1935) and by Lotmar and Picken (1950). Its structure was then modified by Darmon and Rudall (1950). Later, the first detailed structure analysis was that of Carlström (1957) who concurred with an orthorhombic unit cell but obtained different dimensions (Table 1.1). Dweltz (1960, 1961) also proposed a structure for α-chitin using X-ray data. The unit cell contained two polysaccharide chains running in opposite directions and four asymmetric N-acetylglucosamine units. This structure was in agreement with infra-red absorption data.

Evidence for the existence of a second crystalline form of chitin was first obtained by Lotmar and Picken (1950). These authors observed a new X-ray pattern for

Table 1.1 Unit cell dimensions (Å) for α-chitin, β-chitin and γ-chitin

Chitin	a	b	c	References
α	11.58	10.4	19.42	Gonell (1926)
α	9.40	10.46	19.25	Meyer and Pankow (1935)
α	9.25	10.46	19.25	Clark and Smith (1936)
α	9.40	10.26	19.25	Lotmar and Picken (1950)
α	4.76	10.28	18.85	Carlström (1957)
α	4.69	10.43	19.13	Dweltz (1960)
α	4.74 ± 0.01	10.32 ± 0.02	18.86 ± 0.01	Minke and Blackwell (1978)
α	4.71	10.30	18.78	Sikorski et al. (2009)
β	4.7	10.3	10.5	Dweltz (1961)
β	4.85	10.38	9.26	Blackwell et al. (1967)
β (anhydrous)	4.85	10.38	9.26	Blackwell (1969)
β (monohydrate)	4.8	10.4	10.5	Blackwell (1969)
β (dehydrate)	4.8	10.4	11.1	Blackwell (1969)
γ	4.7	10.3	28.4	Walton and Blackwell (1973)

deproteinized pens from the squid *Loligo*. This type form which apparently had a unit cell of dimensions a = 9.32 Å, b = 10.17 Å and c = 22.15 Å, was named β-chitin to distinguish it from the much more common α-chitin. It was found in annelid chaetae (*Aphrodite aculeate*), in the brachiopod *Lingula* and in the skeletal pen of squids (*Loligo*). In arthropods and fungi, only α-chitin appeared to occur. Lotmar and Picken (1950) also noted that the extra-X-ray reflections in the pattern of intact *Aphrodite chaetae* and other tanned material, and suggested that they may be due to ordered protein. Fraenkel and Rudall (1940), studying the X-ray characterization of chitin-protein complexes, previously pointed out the differences between the fiber diagrams of intact and deproteinized insect cuticles. They suggested that the differences were due to modification of the chitin structure due to the presence of complexing protein. Later, Rudall showed that the X-ray patterns of the intact complexes gave many more layer lines than the purified chitin component (Rudall 1955, 1963, 1967). Rudall first remarked that β-chitin was found associated with collagen whereas α-chitin occurred alone or in association with a non-collagenous protein such as arthropodin (Rudall 1955; Richards 1958). He advanced reasons for supposing that the production of α-chitin and collagen may be mutually exclusive. Differences between the two forms of chitin were evidently slight β-chitin being converted to the α-form by dissolving it in anhydrous formic acid or by treatment with strong nitric acid.

Detailed crystallographic investigations have also been reported for the α- and β-forms of chitin by Dweltz (Dweltz 1960, 1961; Dweltz and Anand 1961). Dweltz proposed new crystals structures and comprehensively discussed the spatial configuration of the polymer chain. In both cases, the structures were based upon backbones consisting of straight polysaccharide chains. Basic to the proposed structure for the two systems was the presence of sheets of parallel chains linked by C=O---H-N hydrogen bonds through the amide groups. The forms differed in the sense of the chains in successive sheets. In β-chitin the sheets were all arranged in a parallel manner whereas in the α-form successive sheets were antiparallel. For β-chitin, obtained from the conversion of α-chitin by treatment with formic acid or fuming nitric acid using the experimental protocol published by Lotmar and Picken (1950) and Rudall (1955), Dweltz reported the unit cell to be approximately half that of α-chitin (Table 1.1). For Dweltz, β-chitin must be considered to be a monohydrate in the dry state with the chemical formula $[C_8H_{13}O_5N.H_2O]_n$) and the sugar (glucosamine) in β-chitin was the same as that in α-chitin.

In 1962, Carlström has criticized the structures of α- and β-chitin proposed by Dweltz (Carlström 1962). The main criticism was that the polysaccharide chain configuration was stereochemically unsatisfactory. A straight-chain configuration consisting of glucose units linked together by 1,4-β-glucosidic bonds was sterically impossible. First, Carlström, using the optical transform method, arrived at a new structure for α-chitin (Carlström 1957). An orthorhombic unit cell with a = 4.76 Å, b = 10.28 Å (fibre axis), and c = 18.85 Å was proposed. There were two chitobiose units per unit cell. The repeating period along the fibre axis was the same as that of cellulose. Carlström showed that the chain was in the bent form, similar to that proposed for cellulose (Carlström 1962). This was a major difference in the structure

compared with model proposed by Meyer (Meyer and Mark 1928; Meyer and Pankow 1935). The – $NHCOCH_3$ groups were also assumed to be planar and to be predominantly perpendicular to the fibre axis. An intramolecular hydrogen bond was formed between the carbon-3 hydroxyl group and the ring oxygen of the next acetylglucosamine residue (Carlström 1957, 1962). Carlström proposed a scheme of full intermolecular hydrogen bonding of the C=O---H-N groups along the direction of the a axis. He noted that the hydroxyl group attached to carbon-6 was found to have some rotational freedom. Each glucose residue had a distance between the connecting oxygens of about 5. 45 Å, and this was supported by the excellent crystal structure determination of cellobiose. Carlström (1962) finally reported that optically derived Fourier transforms based on his proposed structure had intensity distributions similar to the observed X-ray intensities.

The same year, Carlström's structure was partially confirmed by Ramachandran and Ramakrishnan (1962). These authors suggested that the data obtained by Dweltz were re-estimated due to differences in the observed intensities. However, they concluded that "it was not possible to say that one structure was superior to the other". The complete intermolecular C=O---H-N hydrogen bonding scheme, originally proposed by Carlström from X-ray data, was also in agreement with the infra-red studies published by Pearson et al. (1960). These authors also reported that there were no free OH and NH groups nor any C=O---H-O bonds in the chitin crystal. With the aid of a scale model of the chitin unit cell, a number of hydrogen-bonding schemes involving the primary hydroxyl groups were proposed. Several attempts were also made over the following 20 years to improve on Carlström's model (Bouligand 1965; Blackwell 1969; Blackwell et al. 1965, 1967, 1980; Neville and Luke 1969a, b; Neville 1970; Ramakrishnan and Prasad 1972; Gardner and Blackwell 1975; Minke and Blackwell 1978).

Another milestone in the discovery of chitin's structure and arrangement was made by Bouligand in 1965 through extensive ultra-structures analyses of crustacean cuticles (Bouligand 1965; Berezina 2016). Bouligand discovered that chitin adopted a stereotypic arrangement (helicoid structure) in arthropods. Thus, three types were also found: α-, β- and γ-chitin. In 1969, Neville and Luke also found that chitin in insect cuticles adopted the Bouligand arrangement as well (Neville and Luke 1969a, b). They suggested that cuticle was arranged in non-lamellate and lamellate systems (Neville and Luke 1969a, b; Neville 1970). The original discovery of cuticle deposition by Bouligand was extensively reviewed and discussed by Neville in the mid-1970s (Neville 1975; Neville et al. 1976).

Blackwell and co-workers reinvestigated the structure of chitin (Blackwell 1969; Blackwell et al. 1965, 1967; Gardner and Blackwell 1975). These authors, studying the crystalline forms of the chitin, reported that the differed in the packing and polarity of the adjacent chain. α-Chitin was the most abundant form and it was found in certain fungi and in arthropod cuticles. α-Chitin was the tightly compacted, most crystalline polymorphic form where the chains were arranged in anti-parallel orientation. Sheets of chains were arranged in stacks along the a axis, the sheets being linked by C=O---H-N hydrogen bonds approximately parallel to the a axis. The strong inter- and intra-molecular bonding leads to the formation of long micro-

fibrils. Blackwell and co-workers also pointed out a number of deficiencies in the model proposed by Carlström (Minke and Blackwell 1978). For α-chitin, their two main criticisms were the following: 1) the CH_2OH side chains were not hydrogen-bonded although infra-red spectroscopic studies showed that all the hydroxyl groups formed donor hydrogen bonds and 2) the presence of two amide I peaks suggested that the arrangement of the amide groups cannot be correct. Marchessault and Sarko (1967) previously reported similar conclusions.

The structure of β-chitin from pogonophore tubes and from the spines of marine diatoms (*Thalassiosira fluviatilis*, *Cyclotella cryptica*) has been refined by rigid-body least-squares methods by Blackwell's group. The β-chitin was the form where the chains were parallel. The unit cell was monoclinic, in agreement with Dweltz' model but different cell dimensions were found (Table 1.1). The increase in the values along the a axis, compared with that for α-chitin, indicated a greater spacing between the chains in this direction. The structure consisted of an array of poly-N-acetyl-D-glucosamine chains all having the same environment, which were also linked together in sheets by C=O---H-N hydrogen bonding of the amide groups. The structure proposed were consistent with the swelling properties of β-chitin and can be seen to be analogous to that of native cellulose (Gardner and Blackwell 1975). Both structures contained extended parallel chains and can be visualized as an array of hydrogen-bonded sheets. β-chitin swelled extensively in water and has been shown to form a series of crystalline hydrates. β-chitin from Polychaetae, when precipitated from acids, also assumed the α-form. In 1988, Blackwell suggested that γ-chitin may be "a distorted version of either α- or β-chitin rather than a true third polymorphic form" (Blackwell 1988).

1.6 The Period of Application: From 1970 Until Now

Despite the significant progress that was made between 1930 and 1970, few researchers at that time believed in the potential that chitin and chitosan had. "Re-discovery" and revived interest in the 1970s encouraged the need to better utilize biowastes from marine crustaceans, due to the introduction of regulation on the dumping of untreated shellfish wastes into the oceans (Muzzarelli 1977; Roberts 1992). From 1970 to 1980, chitin and chitosan entered the period when they reached maturity. Several manufacturers started to produce and to market these products. Chitosan was produced industrially for the first time in Japan in 1971 (Hirano 1989). This industrialization of the production has also contributed enormously to their development. The expansion of the two polysaccharides was also made possible by the first concrete applications in cosmetology, pharmacy, personal care uses, food uses, agriculture, biotechnology, clarification and waste management (sludge dewatering), dentistry and medicine (Feofilova 1984; Zikakis 1984; Hirano 1989; Tsugita 1990; Krajewska 1991; Dodane and Vilivalam 1998). Several patents also claimed the use of chitosan and chitosan derivatives in paper-making (Slagel and Sinkovitz 1973a, b; Plisko et al. 1974).

At the end of 1930s, Kunike was the first to try to produce fibres from chitin (Kunike 1926a, b, c) although the major difficulty was to find a suitable solvent. The discovery of new solvents in the 1970s stimulated new interest in this topic. Research on chitin synthesis, dormant for many years, has also been revived at the end of 1970s due to an unexpected discovery relative to the insecticidal properties of certain benzoylphenyl ureas (Verloop and Ferrell 1977; Hajjar and Casida 1979; Zoebelein et al. 1980). This insecticidal action has generated great interest in insect chitin biosynthesis, in particular in order to contribute to a better understanding of the functional organization of the chitin synthase within the integument and its intricate regulation, and to develop environmentally acceptable pesticides (Hackman 1984, 1987; Cohen 1987a). Two steps in arthropod chitin synthesis have been identified, one sensitive to benzoylphenyl ureas and another to tunicamycin. This clearly discriminated chitin formation in animals from that in fungi and yeasts and demonstrated that different targets for interference besides chitin synthase itself can be used successfully (Roberts 1992; Goosen 1997). At the same time, the potent ability of chitin to accelerate wound healing is discovered (Prudden et al. 1970). Subsequently, many works attempted to implement this discovery in many fields. Today, there are more than 2000 applications of chitin, chitosan and their numerous derivatives (Philibert et al. 2017; see other chapters in this book). These biopolymers continue to offer new horizons to scientists and industrials with a wide range of possible modifications and forms.

In 1973, Riccardo A.A. Muzzarelli edited the first interdisciplinary reference book on polysaccharides entitled "Natural chelating polymers" including three chapters on chitin, chitosan and their analytical applications (Muzzarelli 1973). The same year, Walton and Blackwell (1973), and Brimacombe (1973) comprehensively discussed the structural aspects of chitin and its chemistry. In 1977, Muzzarelli edited another famous chitin sourcebook (Muzzarelli 1977). In that same watershed year, the first international conference on chitin and chitosan was held in Boston in April (11th–13th) organized by Vincent LoCicero of the Massachusetts Science and Technology Foundation, and hosted jointly by the MIT Sea Grant Program and the Massachusetts Science and Technology Foundation. Although the conference was hosted by MIT, the Chairman was RAA Muzzarelli of the University of Ancona, Italy. This first symposium was a great success, with participants coming from all over the world (Muzzarelli and Pariser 1978). The 14th and most recent International Chitin and Chitosan Conference organized by the Japanese Society for Chitin and Chitosan was held in Osaka, Japan (August 27–30, 2018).

Numerous patents have been filed since the 1970s, and an abundant scientific literature has built up. Indeed, a large number of generalist reviews, book chapters and books has been published on practically all the aspects of chitin and chitosan, so many that it would not be feasible to cite them all. I chose to highlight the followings: Jeuniaux (1971, 1982), Brimacombe (1973), Muzzarelli (1973, 1977), Rudall and Kenchington (1973), Walton and Blackwell (1973), Whistler (1973), Neville (1975), Sharon (1980), Cohen (1987a, b), Lezica and Quesada Allué (1990), Roberts (1992), Horst et al. (1993), No and Meyers (1995), Winterowd and Sandford

Table 1.2 Selected books on chitin and chitosan published during the last two decades

Year	Title	References
1997	Applications of chitin and chitosan	Goosen (1997)
2001	Chitin: Fulfilling a biomaterials promise	Khor (2001)
2005	Polysaccharides – structural diversity and functional versatility	Dumitriu (2005)
2009	*Chitine et chitosane – Du biopolymère à l'application*	Crini et al. (2009)
2011	Chitin, chitosan, oligosaccharides and their derivatives: biological activities and applications	Kim (2011)
2011	Chitosan: Manufacture, properties, and usage	Davis (2011)
2011	Chitosan for biomaterials	Jayakumar et al. (2011)
2011	Focus on chitosan research	Ferguson and O'Neill (2011)
2012	Chitosan-based hydrogels: Functions and applications	Yao et al. (2012)
2012	Chitosan-based systems for biopharmaceuticals	Sarmento and das Neves (2012)
2014	Chitin and chitosan derivatives. Advances in drug discovery and developments	Kim (2014)
2015	Advances in marine chitin and chitosan	Sashiwa and Harding (2015)
2016	Chitin and chitosan for regenerative medicine	Dutta (2016)
2017	Chitosan based biomaterials. Fundamentals	Amber Jennings and Bumgardner (2017)
2017	Chitosan – derivatives, composites and applications	Ahmed and Ikram (2017)

(1995), Hudson and Smith (1998), Kurita (1998, 2006), Ravi Kumar (2000), Ravi Kumar et al. (2004), Vårum and Smidsrød (2004a, b), Rauh and Dornish (2006), Rinaudo (2006), Crini and Badot (2008), Peniche et al. (2008), Muzzarelli and Muzzarelli (2009), Renault et al. (2009), Kean and Thanou (2011), Nwe et al. (2011, 2014), Sahoo and Nayak (2011), Teng (2012), Wang et al. (2012), Younes and Rinaudo (2015), Gallo et al. (2016), Bonecco et al. (2017), Dima et al. (2017), Nechita (2017), Ahmed et al. (2018), Akbar and Shakeel (2018), Aljohani et al. (2018), Dimassi et al. (2018), Han et al. (2018), Liaqat and Eltem (2018), Nezakati et al. (2018), Pakdel and Peighambardoust (2018), Pellá et al. (2018), Yu et al. (2018), and Zhao et al. (2018). Table 1.2 shows a selection of books on chitin and chitosan published in the last two decades.

1.7 Conclusion

In this chapter, I have divided the history of chitin into five quite distinct periods, each period being illustrated by relevant references that I have chosen to highlight. The first period, from 1799 to 1894, covers its discovery in fungi by Braconnot in 1811 who called it *fongine*/fungine. The name *chitine*/chitin was conferred by

Odier in 1823 "for what has subsequently been found to be the same compound in insects". The discovery of chitosan, i.e. "*chitine modifiée*", is attributed to Rouget in 1859 but the name chitosan was introduced in 1894 by Hoppe-Seyler. From 1894 to 1930 came a period of confusion due to the controversy about whether or not chitin was identical with cellulose although as early as 1843 both Lassaigne and Payen had noted that chitin contained nitrogen. The third period, from 1930 to 1950, was marked by the characterization of the structure of chitin, by numerous patents and by the first practical applications. These three different periods are illustrated by considering examples of studies that appeared in the literature. From 1950 to 1970 came a short period of doubt although numerous fundamental studies have been published. Finally, the period of real utilization has been in progress since 1970 and has seen chitin and its main derivative, chitosan, find numerous industrial applications.

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Chapter 2
Fundamentals and Applications of Chitosan

Nadia Morin-Crini, Eric Lichtfouse, Giangiacomo Torri, and Grégorio Crini

Abstract Chitosan is a biopolymer obtained from chitin, one of the most abundant and renewable material on Earth. Chitin is a primary component of cell walls in fungi, the exoskeletons of arthropods, such as crustaceans, e.g. crabs, lobsters and shrimps, and insects, the radulae of molluscs, cephalopod beaks, and the scales of fish and lissamphibians. The discovery of chitin in 1811 is attributed to Henri Braconnot while the history of chitosan dates back to 1859 with the work of Charles Rouget. The name of chitosan was, however, introduced in 1894 by Felix Hoppe-Seyler. Because of its particular macromolecular structure, biocompatibility, biodegradability and other intrinsic functional properties, chitosan has attracted major scientific and industrial interests from the late 1970s. Chitosan and its derivatives have practical applications in food industry, agriculture, pharmacy, medicine, cosmetology, textile and paper industries, and chemistry. In the last two decades, chitosan has also received much attention in numerous other fields such as dentistry, ophthalmology, biomedicine and bio-imaging, hygiene and personal care, veterinary medicine, packaging industry, agrochemistry, aquaculture, functional textiles and cosmetotextiles, catalysis, chromatography, beverage industry, photography, wastewater treatment and sludge dewatering, and biotechnology. Nutraceuticals and cosmeceuticals are actually growing markets, and therapeutic and biomedical products should be the next markets in the development of chitosan. Chitosan is also the

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object of numerous fundamental studies. An indication of the widespread exploitation and constantly growing importance of this biopolymer is the total of over 58,625 scientific articles published between 2000 and 2017. In this chapter, after a description of chitosan fundamentals, we highlight selected works on chitosan applications published over the last two decades.

Keywords Chitosan · Chitin · Biopolymer · Fundamentals · Properties · Applications

2.1 Introduction

Main commercial polymers are derived from petroleum-based raw products using processing chemistry that is not always safe or environmental friendly. Over the past three decades, there has been a growing interest in developing natural alternatives to synthetic polymers, namely biopolymers. Biopolymers are polymers derived from living organisms or synthetized from renewable resources. They have expanded significantly due to their biological origin and mostly to their non-toxicity and biodegradable nature. Biopolymers include polysaccharides such as cellulose, starch, chitin/chitosan, and alginates.

Because of its remarkable macromolecular structure, physical and chemical properties, bioactivities, and versatility, quite different from those of synthetic polymers, the biopolymer chitosan has received much attention in fundamental science, applied research and industrial biotechnology (Crini et al. 2009; Kim 2011, 2014; Miranda Castro and Lizárraga Paulín 2012; Teng 2012; Sashiwa and Harding 2015; Dima et al. 2017; Philibert et al. 2017). Chitosan and its derivatives have practical applications in numerous fields: food industry and nutrition, agriculture and agrochemistry, aquaculture, pharmacy, medicine and biomedicine, dentistry, ophthalmology, cosmetology, hygiene and personal care, bio-imaging, veterinary medicine, textile and fiber industries, paper industry, chemistry, catalysis, chromatography, beverage industry, photography, wastewater treatment and sludge dewatering, biotechnology, and nanotechnology (Davis 2011; Ferguson and O'Neill 2011; Sarmento and das Neves 2012; Yao et al. 2012; Bautista-Baños et al. 2016; Dutta 2016; Ahmed and Ikram 2017; Amber Jennings and Bumgardner 2017a, b; Han et al. 2018; Pakdel and Peighambardoust 2018; Pellá et al. 2018; Sharif et al. 2018; Song et al. 2018; Wei et al. 2018; Zhao et al. 2018).

Chitosan is a semi-synthetic commercial aminopolysaccharide derived by deacetylation of the naturally occurring biopolymer chitin. Chitin is the most abundant of the renewable polysaccharides in the marine environment and one of the most abundant on Earth after cellulose (Muzzarelli 1977; Roberts 1992a; Kurita 2006; Rinaudo 2006; Souza et al. 2011). Chitin is an important source of carbon and nitrogen for marine organisms and its ecological importance in the marine environment is nowadays recognized. The main sources exploited for chitin and chitosan

production at industrial scale are marine crustaceans, the shells of shrimps and crabs and the bone plate of squids (Kean and Thanou 2011; Nwe et al. 2011a, b; Kim 2014; Younes and Rinaudo 2015; Elieh-Ali-Komi and Hamblin 2016; Dima et al. 2017; Philibert et al. 2017; El Knidri et al. 2018).

Chitosan extraction from crustaceans in food industry wastes is economically feasible (Truong et al. 2007; Crini et al. 2009). In addition, its commercial production allows the recovery of pigments, proteins, fish meal additive in aquaculture, and also nutraceuticals which are beneficial in human health promotion (Philibert et al. 2017; Dave and Routray 2018). Chitosan can be marketed as a green product. Chitosan is also of great commercial interest due to its high percentage of nitrogen, of 6–7%, compared to synthetically substituted cellulose, of 1.25%, that makes it an effective chelating and complexing agent. Other reasons are undoubtedly its appealing polyelectrolyte properties at acidic pH, and biological properties. Indeed, most of the applications can be related to its cationic nature, which is unique among abundant polysaccharides and natural polymers. Chitosan has unique characteristics such as biocompatibility and biodegradability, and possess reactive functional groups that make is useful in different areas of applications in the form of solutions, suspensions, gels/hydrogels, powders, microparticles/nanoparticles, beads, sponges, foams, membranes and films, or fibers/nanofibers (Muzzarelli 1977; Roberts 1992a; Crini 2005; Kurita 2006; Rinaudo 2006; Crini et al. 2009; Younes and Rinaudo 2015).

The literature on chitosan is vast and spread across different disciplines including chemistry, biochemistry, health science, agriculture and ecology. The past two decades have shown a fast increase in the number of studies on chitosan. Between 2000 and 2017, 58,625 chitosan-related publications have been published including 18,097 on applications (ISI Web of Science database). It is interesting to note that a majority of these works come from Asian nations including Japan (the undisputed leader), Korea, China, Singapore, Taiwan and Thailand (Khor and Lim 2003). A large number of generalist reviews and book chapters has been also published on practically all the aspects of chitosan biopolymer, so many that it would not be feasible to cite them all. Table 2.1 lists selected comprehensive reviews and book chapters on the history, production, characterization, structure, chemistry, derivatives, and toxicology of chitosan. Table 2.2 shows the top ten most cited reviews in the ISI Web of Science database for 2000–2017 with “Chitosan” and “Review” in the topic.

This chapter summarizes some of the developments related to the applications of this biopolymer, based on a substantial number of relevant references, and provides useful information about its most important features. After a brief description of chitosan fundamentals, we present an overview of the applications of chitosan. We include an extensive bibliography of recent studies on chitosan, both basic and applied. Nevertheless, the examples presented are not exhaustive due to the very large number of papers published.

Table 2.1 Selected reviews published during the last two decades on the history, production, characterization, structure, chemistry, derivatives and toxicology of chitosan

General topic	References
Characterization Degree of deacetylation Molecular weight Distribution of N-acetyl groups Spectroscopy: IR, NMR, Raman, X-Ray Elemental analysis Acid-base titration Conductometric/ Potentiometric titration Colloid titration Chemical/Enzymatic hydrolysis	Heux et al. (2000), No and Meyers (2000), Jiang et al. (2003), Shahidi and Abuzaytoun (2005), Wang et al. (2006), Kasaai (2008, 2009, 2011), Crini et al. (2009), Dos Santos et al. (2009), Maniukiewicz (2011), de Alvarenga (2011), Czechowska-Biskup et al. (2012), Zając et al. (2015), Sivashankari and Prabaharan (2017), and Tsai and Chen (2017)
Chemistry Solubility Solution state/Gel state Chain conformation Polyelectrolyte complexes, macromolecular complexes, electrostatic properties Aggregation Self-assembly/Self-assembled systems	Hudson and Smith (1998), Ravi Kumar (2000), Hejazi and Amiji (2001), Kubota and Shimoda (2004), Bodnar et al. (2005), Muzzarelli and Muzzarelli (2005), Shahidi and Abuzaytoun (2005), Crini et al. (2009), Pillai et al. (2009), Hamman (2010), Uragami and Tokura (2010), Jayakumar et al. (2010a), Lakshmanan et al. (2011), Ujang et al. (2011), Rinaudo (2012), Teng (2012), Zambito (2013), Kim and Venkatesan (2014), Mateescu et al. (2015a, b), Younes and Rinaudo (2015), Lizardi-Mendoza et al. (2016), and Salehi et al. (2016)
Derivatives Physical modifications Chemical modifications: derivatization, cross-linking, grafting reactions, hydrolysis Gels/Hydrogels Glucosamines/ Oligosaccharides Chitooligosaccharides/ Chitosan oligomers/ Chitooligomers Technology/Nanotechnology/ Electrospinning Films/Membranes Fibers/Nanofibers	Synowiecki and Al-Khateeb (2003), Canh et al. (2004), Jalal Zohuriaan-Mehr (2005), Wang et al. (2006), Kurita (1986, 1998, 2006), Crini et al. (2009), Muzzarelli and Muzzarelli (2009), Dutta and Dutta (2011), Kim (2011, 2014), Kim and Je (2011), Kim and Kim (2011), Vidanarachchi et al. (2011), Xiao et al. (2012), Yao et al. (2012), Ahmadi et al. (2015), Ahmed and Ikram (2017), Aminabhavi and Dharupaneedi (2017), Ruiz and Corrales (2017), Argüelles-Monal et al. (2018), Divya and Jisha (2018), Liang et al. (2018), and Liaqat and Eltem (2018)
History	Jeuniaux (1966), Muzzarelli (1977), Roberts (1992a), Winterowd and Sandford (1995), Labrude (1997), Labrude and Becq (2003), Shahidi and Abuzaytoun (2005), Rauh and Dornish (2006), Wisniak (2007), Crini et al. (2009), Khoushab and Yamabhai (2010), Nwe et al. (2011b), Muzzarelli et al. (2012), Berezina (2016), Annu et al. (2017), and Karthik et al. (2017)

(continued)

Table 2.1 (continued)

General topic	References
Production Sources Industrial production Extraction: chemical, biological, enzymatic Microorganisms Chitin deacetylases	Viney and Harish (2002), Synowiecki and Al-Khateeb (2003), Sen (2005), Kurita (2006), Abdou et al. (2008), Peniche et al. (2008), Crini et al. (2009), Brück et al. (2011), Davis (2011), Jo et al. (2011), Knezevic-Jugovic et al. (2011), Nwe et al. (2011a, b, 2013, 2014), Arbia et al. (2013), Gortari and Hours (2013), Setyahadi (2013), Kaur and Dhillon (2014, 2015), Aljawish et al. (2015), Younes and Rinaudo (2015), Majekodunmi (2016), Sivashankari and Prabaharan (2017), Dave and Routray (2018), de Lima Batista et al. (2018), El Knidri et al. (2018), and Grifoll-Romero et al. (2018)
Structure Structural aspects/ Morphology/Crystallinity Crystallography Structure-property relationship	Vårum and Smidsrød (2004a), Dutta et al. (2004), Kurita (2006), Rinaudo (2006), Dash et al. (2011), Younes and Rinaudo (2015), and Sahariah and Másson (2017)
Toxicology Toxicity Cytotoxicity Biodegradation	Kurita (1998), Pillai et al. (2009), Kean and Thanou (2010), Dash et al. (2011), Jimtaisong and Saewan (2014), Sahariah and Másson (2017), and Sadler and Funnell (2018)

Table 2.2 The top ten most cited reviews in the ISI Web of Science database for 2000–2017 with "Chitosan" AND "Review" in the topic; out of a total of 2702 reviews appearing, October 01, 2018

1. Rinaudo M (**2006**) Chitin and chitosan: Properties and applications. Progress in Polymer Science 31:603–632. Times cited: 3092.
2. Kumar MNVR (**2000**) A review of chitin and chitosan applications. Reactive and Functional Polymers 46:1–27. Times cited: 2297.
3. Crini G (**2006**) Non-conventional low-cost adsorbents for dye removal. Bioresource Technology 97:1061–1085. Times cited: 2229.
4. Rabea EI, Badawy MET, Stevens CV, Smagghe G, Steurbaut W (**2003**) Chitosan as antimicrobial agent: Applications and mode of action. Biomacromolecules 4:1457–1465. Times cited: 1401.
5. Agnihotri SA, Malikarjuna NN, Aminabhavi TM (**2004**) Recent advances on chitosan-based micro- and nanoparticles in drug delivery. Journal of Controlled Release 100:5–28. Times cited: 1388.
6. Suh JKF, Matthew HWT (**2000**) Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: A review. Biomaterials 21:2589–2598. Times cited: 1221.
7. Berger J, Reist M, Mayer JM, Felt O, Peppas NA, Gurny R (**2004**) Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. European Journal of Pharmaceutics and Biopharmaceutics. 57:19–34. Times cited: 1120.
8. Dash M, Chiellini F, Ottenbrite RM, Chiellini E (**2011**) Chitosan – A versatile semi-synthetic polymer in biomedical applications. Progress in Polymer Science 36:981–1014. Times cited: 1110.
9. Shahidi F, Arachchi JKV, Jeon YJ (**1999**) Food applications of chitin and chitosans. Trends in Food Science Technology. 10:37–51. Times cited: 1066.
10. Khor E, Lim LY (**2003**) Implantable applications of chitin and chitosan. Biomaterials 24:2339–2349. Times cited: 1053.

2.2 Chitosan Production and Properties

2.2.1 History

In 1811, Braconnot, Director of the *Jardin Botanique* of Nancy, France, discovered an alkaline-insoluble fraction from mushrooms by treatment with dilute warm alkali (Simonin 1856, 1870, 2013; Prévost and D'Amat 1956; Tracey 1957; Jeuniaux 1966; Muzzarelli 1977; Roberts 1992a; Winterowd and Sandford 1995; Labrude 1997; Labrude and Becq 2003; Wisniak 2007). Braconnot described this new product containing a substantial percent of nitrogen and found that the liquid contained acetate of ammonia contaminated with oil (Braconnot 1811, 1813). This fraction also produced acetic acid by degradation with concentrated sulfuric acid (Braconnot 1811; Tracey 1957; Rudall and Kenchington 1973). Braconnot gave the name alkaline-insoluble fraction as *fongine*/fungine, a substance "*d'une nature particulière*".

In 1823, Odier isolated a similar alkaline-insoluble fraction from the elytra of insects by repeated treatments with hot hydroxide solutions (Odier 1823). He gave it the name of *chitine*/chitin, from Greek word chitos, meaning tunic or envelope (Odier 1823; Rudall and Kenchington 1973; Souza et al. 2011). However, Odier tested for nitrogen in this residue and found none. Odier's paper was promptly published in an English translation by Children in 1824 (Children 1824). Children, repeating the same experiments, also extracted an alkaline-insoluble fraction from may bug elytra and found the presence of nitrogen by elemental analysis (Children 1824; Tracey 1957; Nwe et al. 2011b).

In 1843, Lassaigne isolated a chitinous material from treatment of exoskeleton of silkworm butterfly and confirmed the presence of nitrogen in chitin (Lassaigne 1843). He proved that chitin was not a cellulose but a new product containing nitrogen (Tracey 1957). The same year, the Lassaigne's finding were corroborated by Payen (1843), although initially he had convinced himself in 1840 that Braconnot's fungine was cellulose (Tracey 1957).

In the 1870s, Ledderhose hydrolyzed arthropod chitin with the aim to identify the structure of the products (Ledderhose 1876, 1878). In 1875, Ledderhose, treating lobster shells with hot concentrated hydrochloric, found that the shells dissolved in this solution and that on evaporation the solution yielded characteristic crystals. He identified the crystalline compound as a new amino-containing sugar, which he named *glykosamin*/glucosamine (Ledderhose 1876). Later, Ledderhose showed that acetic acid was also a product of hydrolysis of arthropod chitin (Ledderhose 1878). The presence of glucosamine as the repeated unit of chitin was confirmed a few years later by the works of Winterstein (Winterstein 1893, 1894) and Gilson (Gilson 1894, 1895). At the ends of the 1890s, it was established that chitin occurs in both animals and plants and that there was no detectable chemical differences between products from the two sources. However, the controversy of the structure of chitin has continued.

The history of chitosan dates back to 1859 with the work of Rouget (Rouget 1859; Brimacombe and Webber 1964; Muzzarelli 1977; Sharon 1980; Roberts 1992a; Winterowd and Sandford 1995). Rouget treated chitin with strong alkali, which resulted in a new substance that could, unlike chitin itself, be dissolved in acidic aqueous solutions (Rouget 1859). He named the product "*chitine modifiée*", modified chitin. In 1894, Hoppe-Seyler treated the shells of crabs, scorpions and spiders with potassium hydroxide at 180 °C and found a "new" product (Hoppe-Seyler 1894). He gave it the name of chitosan. This product was a partially deacetylated, acid-soluble derivative of chitin. This was also prepared from fungal material by both Winterstein (1894) and Gilson (1894).

In the middle of the 1930s, the structure of chitin and chitosan was characterized using data from X-ray diffraction (Meyer and Pankow 1935; Clark and Smith 1936) and later by enzymatic analysis and infrared spectroscopy (Muzzarelli 1977; Roberts 1992a). In 1936, there was the first use in papermaking industry. In the same year, two patents, one for producing chitosan from chitin and the other for making films and fibers from chitosan, were obtained by Rigby. However, lack of adequate manufacturing facilities and mostly cutthroat competition from synthetic polymers restricted their commercial development. "Re-discovery" and revived interest in the 1970s encouraged the need to better utilize biowastes from marine crustaceans (Muzzarelli 1977; Roberts 1992a, 1998; Winterowd and Sandford 1995; Rauh and Dornish 2006; Wisniak 2007; Crini et al. 2009; Khoushab and Yamabhai 2010; Nwe et al. 2011b). Several manufacturers started to produce and to market chitin and chitosan products. Numerous patents have been filed since the 1970s, and an abundant scientific literature has built up (Muzzarelli et al. 2012; Berezina 2016; Annu et al. 2017; Karthik et al. 2017). Today, there are more than 2000 applications of chitin, chitosan and their numerous derivatives (Philibert et al. 2017).

2.2.2 Chitin

Among numerous biopolymers, chitin is produced in the largest amounts, and after cellulose, it is the most abundant organic compound from living organisms on Earth. This polysaccharide is widely distributed in the animal and vegetal kingdom, constituting an important renewable resource. Chitin is found in the exoskeleton of crustaceans, the cuticles of insects, and the cell walls of fungi (Rudall and Kenchington 1973; Muzzarelli 1977; Muzzarelli et al. 1986a; Roberts 1992a; Kurita 1986, 1998, 2006; Rinaudo 2006; Nwe et al. 2011b). Other examples are given in Table 2.3. The content of chitin is 30–40% in shrimps, 15–30% in crabs, 20–30% in krills, 20–40% in squids, 3–6% in clams, 3–6% in oysters, 5–25% in insects and 10–25% in fungi (Muzzarelli et al. 1986a; Skjåk-Braek et al. 1989; Roberts 1992a; Horst et al. 1993; Felt et al. 1998; Kurita 2006; Rinaudo 2006; Nwe et al. 2011b). A discussion on chitin content can be found in the recent review by Hamed et al. (2016).

From a structural point of view, both chitin and cellulose are polymers of monosaccharide made up of β-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranose and

Table 2.3 Sources of chitin and chitosan in aquatic organisms, terrestrial organisms and microorganisms

Aquatic crustaceans	Other sea animals	Insects	Mushrooms and Fungi	Other microorganisms	Other terrestrial crustaceans
Shrimps Crabs Lobsters Crayfishes Krill	Squids Cuttlefishes Annelida Mollusca Coelenterata	Scorpions Spiders Brachiopods Cockroaches Beetles Silkworms Ants Mosquitoes Honey bees	*Agaricus bisporus* *Auricularia auriculajudae* *Lentinula edodes* *Trametes versicolor* Blastocladiaceae Ascomydes *Aspergillus niger* *Mucor rouxii*	Green algae Brown algae Yeast Spores	Nematode *Porcellio scaber* *Armadillidium vulgare*

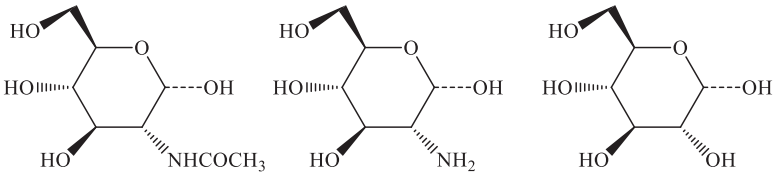

Fig. 2.1 Structure of (**a**) N-acetyl-glucosamine, monomer of chitin, (**b**) glucosamine, monomer of chitosan and (**c**) glucose, monomer of cellulose

β-(1 → 4)-2-deoxy-β-D-glucopyranose units, respectively (Fig. 2.1). Chitin is generally represented as a linear long-chain homo-polymer composed of N-acetyl glucosamine units, [poly(N-acetyl-β-D-glucosamine)] (Fig. 2.2). Depending on its source, chitin exists in three different polymorphic forms, namely α-, β- and γ-chitin (Bouligand 1965; Neville and Luke 1969a, b; Neville 1975; Neville et al. 1976; Muzzarelli 1977; Roberts 1992a; Horst et al. 1993; Berezina 2016), which can be differentiated by X-ray diffraction, and infrared and NMR spectroscopy (Muzzarelli 1977; Roberts 1992a; Maniukiewicz 2011). α-Chitin isomorph is by far the most abundant. The sources for α-, β- and γ-chitin are crabs (e.g. *Callinectes sapidus*, *Chionoecetes opilio*) and shrimps (*Pandalus borealis*, *Crangon crangon*), squids and loligo (*Ommastrephes bartrami*, *Loligo formosana* Sasaki), respectively. These forms differ in their arrangement of macromolecular chain. In α-chitin, the chains are arranged antiparallel to each other while in β-chitin they are parallel. In γ-chitin, the chains are arranged randomly in which two parallel chains and one antiparallel chain form the structure. An update discussion on this topic can be found in the references by Maniukiewicz (2011), Anitha et al. (2014), and Younes and Rinaudo (2015).

Chitin is structurally similar to cellulose: it shares many structural similarities as, for example, the conformation of the monomers and the diequatorial glycosidic

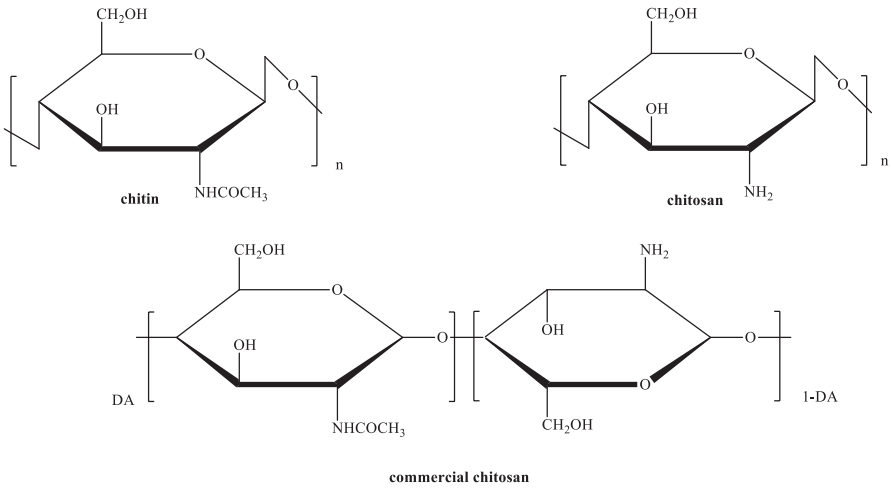

Fig. 2.2 Schematic representation of completely acetylated chitin [poly(*N*-acetyl-ß-D-glucosamine], completely deacetylated chitosan [poly(D-glucosamine] and commercial chitosan, a copolymer characterized by its average degree of acetylation (DA) per cent

linkages. Both biopolymers play similar roles since they both act as structural support and defense materials in living organisms. However, chitin is an aminopolymer and has acetamide groups at the C-2 positions in place of the hydroxyl groups in the repeating unit of macromolecular chains (Figs. 2.1 and 2.2). Chitin is also an extremely insoluble material (Austin 1984; Kienzle-Sterzer et al. 1984; Muzzarelli 1977; Muzzarelli et al. 1986b; Skjåk-Braek et al. 1989; Roberts 1992b, c; Hudson and Smith 1998). Despite its huge availability, its insolubility is a major problem that confronts the development of processes and uses of this chitin, and so far, few large-scale industrial uses have been found. For instance, chitin is an effective material for sutures because of its biocompatibility, non-toxicity, biodegradability, antimicrobial activity and low immunogenicity. Chitin also accelerates wound-healing in spray, gel and gauze and it can be used to control drug release (Pillai et al. 2009; Younes and Rinaudo 2015).

More important that chitin is its main derivative, chitosan. Indeed, partial deacetylation of chitin results in the commercial production of chitosan which is a polysaccharide composed by macromolecules of D-glucosamine and N-acetyl glucosamine (Hudson and Smith 1998). The fully deacetylated product is rarely obtained due to the risks of side reactions and chain depolymerization. Chitosans may be considered as a family of linear binary copolymers of β-(1 → 4)-linked N-acetyl glucosamine units and 2-amino-2-deoxy-β-D-glucose, and do not refer to a uniquely defined polysaccharide, but to products having different proportions of D-glucosamine and N-acetyl glucosamine units and of varying chain lengths. In fact, the “chitosan label” generally corresponds to polymers with less than 25–40% acetyl content. So, each commercial chitosan is a copolymer characterized by its average degree of acetylation (DA) per cent (Fig. 2.2). This degree of acetylation is

defined as the percentage DA or fraction F_A of N-acetylated glycosidic units in chitosan or chitin biopolymer. It is also customary to express this parameter as degree of deacetylation DD (= 1-F_A or = 100-DA per cent). In chitin samples, the degree of acetylation is typically 90% indicating the presence of some amino groups, as some amount of deacetylation might take place during chitin extraction. Depending on the source, a variable and small proportion of these units are also deacetylated in natural chitin (Peniche et al. 2008). However, this remains always a subject of debate (Peniche et al. 2008; Pillai et al. 2009; Younes and Rinaudo 2015).

2.2.3 Production

Although chitin is found in many aquatic organisms, in many insects, in terrestrial crustaceans, in mushrooms, and in some fungi, from a production point of view, the raw biopolymer chitin is only commercially extracted from marine crustaceans primarily because a large amount of wastes/biowastes is available as low-cost by-products of seafood processing industry (Truong et al. 2007; Nwe et al. 2011b, 2014; Dave and Routray 2018). Shrimps, crabs, lobsters, krill, and squid biowastes from marine industry are the major resources used for the large-scale production of chitin and chitosan. The shells of these marine crustaceans contain 15–40% chitin, proteins (20–40%) and calcium carbonate (20–50%) being two other major components. Pigments, lipids and other minerals including metals and salts are minor components. Of course, the percentages vary considerably with the species used (quality and freshness of shell) and the season (Peniche et al. 2008; Nwe et al. 2014). Other possible sources from marine organisms of chitin production include clams and oysters. Figure 2.3 shows a simplified representation of preparation of chitin, chitosan and their derivatives from aquatic crustacean biowastes. The shells are first ground to the appropriate size and washed profusely with water to remove any organic material adhered to their surface (Acosta et al. 1993). To isolate chitin from the shells of shrimps and crabs, and bone plates of squids, three main steps are then required, namely deproteinization, demineralization, and decolorization. The order of the first two steps is considered irrelevant if protein or pigment recovery is not an objective (Peniche et al. 2008; Nwe et al. 2011b).

The conventional protocol for chitin production is performed through chemical processes using inorganic strong alkali for protein extraction at a given temperature, usually 50–60 °C, and acids for removal of inorganic components, and oxidants for decolorization. These processes permit to dissolve inorganic salts, mainly calcium carbonate and calcium phosphate, using hydrochloric acid and proteins using sodium hydroxide, to eliminate lipids and pigments (melanins, carotenoids), and to obtain a colorless commercial product. The considerable quantities of carotenoids can be used as fish food additives. Calcium carbonate can be converted to calcium oxide and sodium carbonate. The proteins extracted can be recovered by lowering the pH of the solution to its isoelectric point for precipitation. The recovered proteins can be also used as a high-grade additive for livestock starter feeds. This

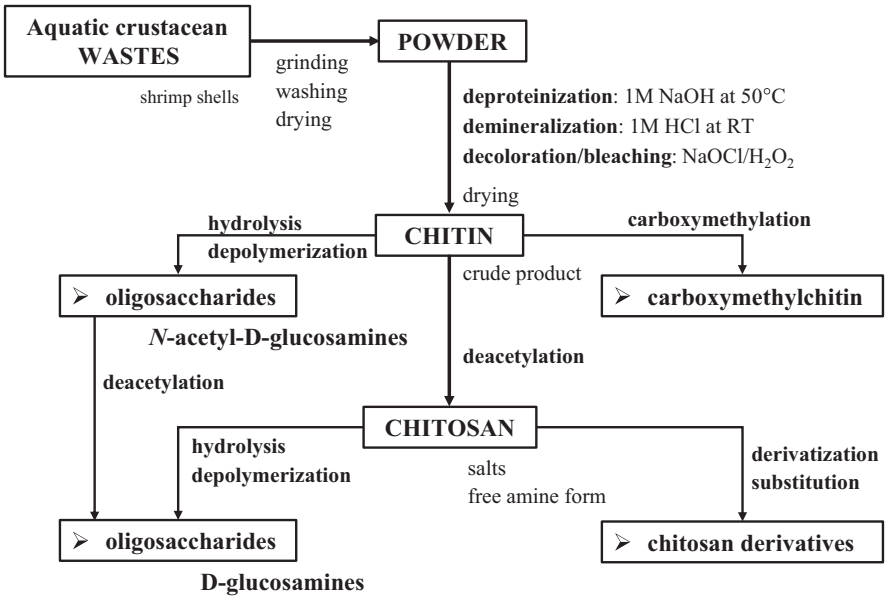

Fig. 2.3 Simplified representation of preparation of chitin, chitosan and their derivatives from aquatic crustacean biowastes

decreases the manufacturing costs of chitin. Calcium carbonate is usually eliminated with dilute HCl solutions at room temperature although other acids have been also studied with success. The acid concentration, reaction time and temperature must be carefully controlled in order to minimize the hydrolytic depolymerization and deacetylation of chitin and/or to prevent thermal degradation (No and Meyers 1995; Percot et al. 2003; Peniche et al. 2008). The color of crustacean shells is due to the presence of pigments such as astaxanthin, cantaxanthin, astacene, lutein and β-carotene (Acosta et al. 1993; No and Meyers 1995; Kurita 1998). Decolorization/bleaching is achieved through the use of potassium permanganate or sodium hypochlorite (Kurita 1998, 2006) or by solvent extraction (Acosta et al. 1993; Peniche et al. 2008). Oxidation may attack the free amino groups and introduce modifications in the macromolecules (Acosta et al. 1993). Pigments may be also recovered as high value side products.

Purified chitin is then converted to chitosan by hydrolysis of acetamide groups of chitin using sodium hydroxide solutions 40%–60% v/v (No and Meyers 1995; Kurita 1998). The deacetylation process de-esterifies the N-acetyl linkages. Generally, the reaction is carried out under N_2 atmosphere or in the presence of a scavenger of oxygen to avoid depolymerization and to prevent chain degradation. The specific reaction conditions depend on several factors such as the origin of the raw material, the previous treatment and the desired degree of acetylation. Deacetylation of chitin is undertaken using homogeneous or heterogeneous processes (Fig. 2.4). Homogeneous deacetylation is achieved with more moderate

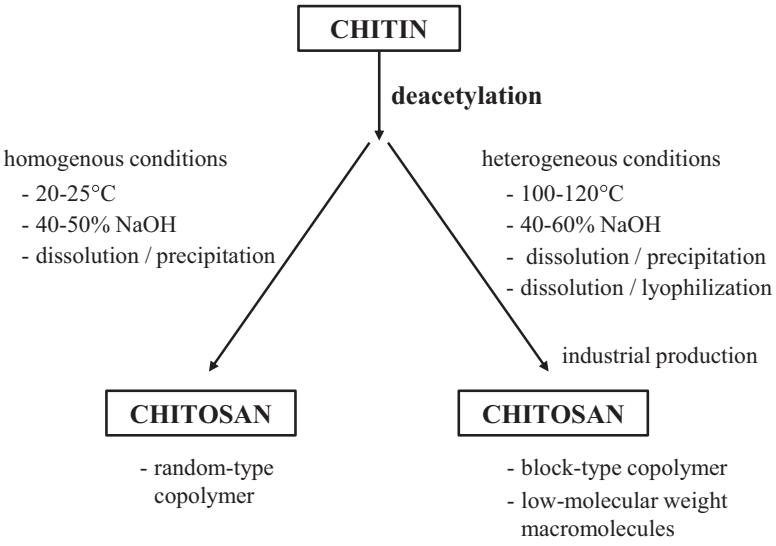

Fig. 2.4 Deacetylation of chitin using homogeneous and heterogeneous processes

alkali concentrations at room temperature and chitosans obtained do not exhibit chain compositional dispersion while chitosans obtained by the heterogeneous process are polydispersed in terms of degree of acetylation of their chains and distribution. High temperatures are preferred for industrial chitosan production (Kurita 1986, 1998, 2006). The chitosan obtained is finally dried into flakes, and then dissolved in acetic acid and filtered to remove extraneous materials resulting in a water-soluble salt. To purify it, the product is dissolved in excess acid and filtered using porous membranes. Adjusting the pH of the solution to ca. 7.5 by NaOH causes flocculation due to the deprotonation and the insolubility of the macromolecule chains at neutral pH. The sample is then washed with water and dried. Data published on worldwide chitosan market is estimated to be over 40,000 metric tons in 2018 (Philibert et al. 2017).

The chemical methods for chitin/chitosan production from biowastes are widely used for industrial purposes due to their low cost and suitability to mass production (Nwe et al. 2014; Younes and Rinaudo 2015). However, their production leads to inconsistent physicochemical characteristics of products, e.g. different grades of quality, protein contamination, different levels of degree of acetylation and inconsistent molecular weight, because of seasonal, climate and variable supply of raw materials, e.g. quality of the shell, species nature, as well as macromolecular variability and difficulties of process conditions: long processing times, toxicity of reactants, as previously reported by No and Meyers (1995). This results in a wide unpredictable range of properties and this is a major concern for the use of crustacean chitosan in the medical and pharmaceutical fields.

Another often cited issue is the fact that the extraction processes are not ecological. The industrial methods consume high amounts of water and energy, use large

amounts of alkali, and generate large quantities of hazardous chemical wastes. To overcome these problems, alternatives to the chemical production of chitin and chitosan are the biological treatments such as microbial production and enzymatic hydrolysis (Kurita 1998, 2006; Kim 2011; Jo et al. 2011; Philibert et al. 2017). Actually, the biotechnology fermentation process of chitin from crustacean wastes is proposed on an industrial scale. However, the proteins and minerals are not completely removed by the biological treatment. Other drawbacks are a lower quality and a higher cost compared with the industrial, chemical process. The fermentation processes offer, however, new perspectives for the production of chitosan. Microorganisms and terrestrial organisms like insects, terrestrial crustaceans, and mushrooms were also considered as alternative sources for the large-scale production of chitin and chitosan. In particular, the biotechnological production of chitin/chitosan from microbial sources appears promising because the products obtained are pure with specific characteristics. However, the yield of purified chitin and chitosan from these sources is lower than that of aquatic crustacean sources. Different preparation methods often result in differences in the degree of acetylation, distribution of acetyl groups, chain length (molecular weight) and conformational structure of chitin and chitosan. In addition, although significant efforts have been made to optimize the fermentation process for the production of these two biopolymers, the technology may still be significantly improved. For more details on the chitin/chitosan production, the following reviews can be consulted: Muzzarelli et al. 1986a; Skjåk-Braek et al. 1989; Horst et al. 1993; Kurita 1998; Rinaudo 2006; Nwe et al. 2011a, b, 2013, 2014; Younes and Rinaudo 2015; Philibert et al. 2017.

2.2.4 Nomenclature of Chitin and Chitosan

Nowadays, chitosan and chitin are known as copolymers of D-glucosamine and N-acetyl-D-glucosamine units, characterized by their average degree of acetylation or degree of deacetylation. However, the nomenclature of chitin and chitosan remains always a subject of debate. Until the end of 1990s, it was agreed to define chitin and chitosan as polymers with degree of acetylation more than 40% and less than 40%, respectively (Peters 1995). In the 2000s, Eugene Khor (National University of Singapore) defined that "when the number of N-acetyl glucosamine units is higher than 50%, the biopolymer is termed chitin; conversely, when the number of D-glucosamine units is higher, the term chitosan is used" (Khor 2001; Khor and Lim 2003). In 2008, George AF. Roberts (Nottingham University, UK) proposed to develop a nomenclature system and to characterize chitin and chitosan with their mole fraction of D-glucosamine or N-acetyl glucosamine (source: European Chitin Society). In 2011, Nitar Nwe (Kansai University, Japan) also proposed to develop a systematic nomenclature system for chitin and chitosan products (Nwe et al. 2011a, b).

Chitin and chitosan can be also defined on the basis of their differences in solubility in water. Chitosan is soluble in aqueous acetic acid while chitin is insoluble

and also in the vast majority of common solvents. This is the reason why chitin was considered an intractable polymer and for many years, it remained mainly a laboratory curiosity (Peniche et al. 2008). Based on the solubility properties of products in acid and alkaline solutions, the name of chitin and chitosan was previously given by Odier in 1823 and Hoppe-Seiler in 1894, respectively. Because of its high cohesive energy related to strong intra- and intermolecular interactions through hydrogen bonds, commercial chitin like cellulose is difficult to dissolve.

Chitin also possesses a low content of amino groups, and therefore, its macromolecules are not soluble in water and do not swell in common solvents, except β-chitin. Chitosan has a rigid crystalline structure through intra- and inter-molecular hydrogen bonding. However, it is soluble in acidic media, except in benzoic acid and in oxalic acid. Indeed, in contrast to chitin, the presence of free amino groups on the C-2 position of the D-glucosamine unit along the macromolecule chains allows chitosan to dissolve in dilute aqueous acidic solvents through the protonation of these groups and the formation of the corresponding polymer salt. The biopolymer is then converted to a polyelectrolyte in acidic media. Nowadays, the nomenclature described in Table 2.4 is accepted. However, as discussed by Nwe and collaborators, "Do all type of chitosans with 45-50% degree of acetylation dissolve in water?" The answer to this question is not yet clear (Nwe et al. 2011b).

2.2.5 Characterization

Each commercial batch of chitosan is characterized by its degree of acetylation or degree of deacetylation, molecular weight, and others specific characteristics such as appearance of product, purity, crystallinity, solubility, turbidity of polymer solution, ash content, etc. (Nwe et al. 2014; Younes and Rinaudo 2015). These characteristics are of major importance on applications of these products. Several reviews can be consulted on these different aspects of chitosan characterization (Table 2.1). Commercial industrial or technical chitosans are usually offered as flakes or powders with prices ranging from 15 to 100 US$/kg, depending mainly on degree of

Table 2.4 Nomenclature of chitin and chitosan

Product	Characteristics	Degree of deacetylation	Molecular weight
Chitin	Insoluble in alkaline solution Insoluble in dilute organic acids	>50%	>5 kDa
Chitosan	Insoluble in alkaline solution Soluble in dilute organic acids Precipitation in solution with alkaline pH	<50%	>5 kDa
Chito-oligosaccharide Oligochitosan	Soluble in alkaline solution Soluble in acid solution Soluble in neutral media	All	<5KDa

acetylation/degree of deacetylation and purity parameters and specifications of products, e.g. appearance, viscosity, ash content, and metal content lower than 10 mg/kg. An ultrapure grade chitosan, e.g. with low levels of heavy metals, total metals, bacteria, ash, and of protein, and with no bleaching step during extraction and purification processes, for biomedical applications can cost upward of US$ 20,000/kg (Fig. 2.5). Several companies also offer chitosan as bead forms.

The main characteristic of a commercial sample is its average degree of acetylation. The most convenient method for the determination of the degree of acetylation for soluble samples is ^{1}H-NMR spectroscopy (Hirai et al. 1991; Vårum et al. 1991, 1992; Rinaudo 2006; Kasaai 2008, 2009, 2011). The value is calculated from the integral ration between proton of acetyl group of N-acetyl glucosamine and the protons of D-glucosamine. Other methods include solid state NMR, FT-IR, Raman spectroscopy, UV spectroscopy, circular dichroism, potentiometry, conductimetry, hydrolytic and enzymatic techniques (Focher et al. 1992; Raymond et al. 1993; Rinaudo et al. 1997; Peniche et al. 2008; de Alvarenga 2011). The degree of acetylation of a sample is an important factor influencing its physical and chemical properties: solubility, flexibility and polymer conformation, viscosity, crystallinity, conductivity, tensile strength and photoluminescence. It also influences the biological properties of chitosan: biodegradability, biocompatibility, mucoadhesion, hemostatic, analgesic, adsorption enhancer, antimicrobial, anticholesterolemic, and antioxidant.

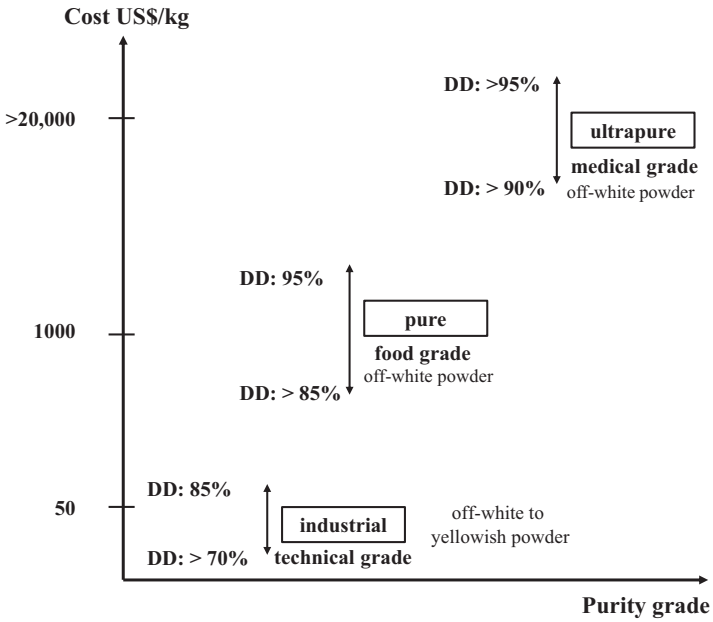

Fig. 2.5 Cost-purity relationship of chitosans (DD: degree of deacetylation)

Another important characteristic to consider is the molecular weight of each sample, after dissolution, and its distribution. This parameter influences on the viscosity of aqueous solution of chitosan and has also a significant role not only on the chitosan properties but also in the industrial applications in many fields. For example, in medical and agricultural domains, low-molecular weight chitosans are preferred (Nwe et al. 2011b). The most relevant technique used for molecular weight determination is viscosimetry. Other techniques such as gel permeation chromatography, light scattering and osmometry (Rinaudo et al. 1997; Peniche et al. 2008; Kasaai 2011) can be used. Polysaccharides are in general polydisperse with respect to molecular weight, and chitosan products are no exception. The molecular weight of a sample is thus an average over the whole distribution of molecular weights. Each sample can be characterized by a polydispersity index (Rinaudo 2006; Peniche et al. 2008). An important step in chitosan characterization is also the distribution of acetyl groups along the macromolecular chain. Indeed, this may influence the solubility of the sample and the inter-chain interactions (Younes and Rinaudo 2015). This distribution can be evaluated using ^{13}C-NMR spectroscopy (Vårum et al. 1991, 1992; Kasaai 2011).

The degree of acetylation and molecular weight are undoubtedly the most important parameters to establish the physical and chemical identity of chitin and chitosan products. These parameters have an important effect of the physical, chemical and biological properties of products. However, both parameters vary with the source of the raw material and the preparation method. In addition, they dictate the functional and technological properties of these biopolymers. Other properties such as solubility, viscosity, and gelling capacity are also dependent on these two parameters (Gunjal et al. 2004; Rinaudo 2006; Nwe et al. 2011a, b, 2014).

2.2.6 Properties

Chitosan presents multiple physical-chemical, biological and technological properties as reported in Table 2.5. The main property of chitosan is its cationic nature and its particular behavior in solution. Indeed, this biopolymer is the only natural cationic polymer in the nature (Muzzarelli and Muzzarelli 2005; Rinaudo 2006). Figure 2.6 illustrates chitosan's versatility in aqueous solution. At low pH, usually less than about 6.3, chitosan's amine groups are protonated conferring polycationic behavior to polymer while at higher pH (above 6.3), chitosan's amine groups are deprotonated and reactive. Usually, chitosan becomes soluble in all aqueous acidic media when the average degree of acetylation is lower than 50%. Chitosan is soluble in dilute inorganic acids such as HCl, HBr, HNO_3 and $HCLO_4$, in concentrated H_2SO_4, in organic acids, e.g. acetic, citric, formic, lactic, and in organic solvents: tetrahydrofuran, ethyl-acetate, 1,2-dichlorethane. Acetic and formic acids are the most commonly used for solubilization of chitosan. However, the solubility is a difficult parameter to control because it is related to the degree of acetylation, the ionic concentration, the pH of the solution used, the nature of the acid used for

Table 2.5 Intrinsic physical-chemical, biological and technological properties of chitosan

Physical-chemical properties	**Selected references**
Biopolymer with rigid D-glucosamine structure containing numerous amino/hydroxyl groups Linear amino-polysaccharide with high nitrogen content, hydrophilicity and high crystallinity Two grades of high and low molecular weight Three main grades of purity: technical, pure and ultra-pure Reactive amino/hydroxyl groups for physical modification, chemical activation or enzymatic modification: high reactivity Weak base, powerful nucleophile: pK_a ~ 6.3 Soluble in dilute acidic aqueous solutions Insoluble in water and organic solvents Forms salts with organic and inorganic salts Water retention Capacity to form hydrogen bonds and chemical interactions Aggregation behavior Electrostatic, chelating and complexing properties Surface active properties Ionic conductivity Rheological behavior	Muzzarelli et al. (1986a, b), Roberts (1992a), Winterowd and Sandford (1995), No and Meyers (1995), Kim et al. (1999), Viney and Harish (2002), Vårum and Smidsrød (2004b), Yilmaz (2004), Shahidi and Abuzaytoun (2005), Rinaudo (2006), Rauh and Dornish (2006), Peniche et al. (2008), Elsabee et al. (2009), Sahoo and Nayak (2011), Teng (2012), Sashiwa and Harding (2015), Annu et al. (2017), and Tripathi and Singh (2018)
Biological properties	**Selected references**
Non-toxicity Biocompatibility; not digestible by humans: dietary fiber Biodegradability Hydrating agent Encapsulating material, microencapsulation Delivery systems and drug releasing activity; carriers and immunoadjuvants in vaccine delivery Bioactivities: analgesic effect, antimicrobial (antibacterial, antifungal), antioxidant, anti-inflammatory, anti-acid, antiulcer, anticoagulant, antihypertensive, hypolipidemic, antidiabetic, anticancer, antitumor, anti-HIV, bioadhesivity Abilities: healing, self-healing, wound healing; mucoadhesion, bone regeneration, immuno-adjuvant	Peniche et al. (2003), Rabea et al. (2003), Vårum and Smidsrød (2004b), Shahidi and Abuzaytoun (2005), Rinaudo (2006), Goy et al. (2009), Friedman and Juneaj (2010), Dash et al. (2011), Kim and Kim (2011), Vyas et al. (2011), Ta et al. (2011), Dutta and Dutta (2011), Je and Ahn (2011), Yamazaki and Hudson (2012), Fernandez-Saiz (2012), Estevinho et al. (2013), Karadeniz and Kim (2014a, b), Moratti and Cabral (2017), Tachaboonyakiat (2017), Verlee et al. (2017), Vunain et al. (2017), Jardine and Sayed (2018), Perinelli et al. (2018), Sharif et al. (2018), Singh et al. (2018), Tripathi and Singh (2018), and Vasconcelos and Pomin (2018)

(continued)

Table 2.5 (continued)

Technological properties	Selected references
Polyelectrolyte at acidic pH; cationic biopolymer with high charge density at pH < 6.3: one positive charge per glucosamine residue Interaction with negatively charged molecules; adheres to negatively charged surfaces Adhesivity/Bioadhesivity Film-forming ability Versatility: solutions, biosorbent, fibers, films Gelation ability; viscosity: high to low Coagulating agent / Flocculating agent Entrapment and adsorption properties Filtration and separation Materials for isolation of biomolecules	Muzzarelli et al. (1986b), Sandford (1989), No and Meyers (1995), Kim et al. (1999), Vårum and Smidsrød (2004b), Shahidi and Abuzaytoun (2005), Hamman (2010), Mati-Baouche et al. (2014), and Younes and Rinaudo (2015)

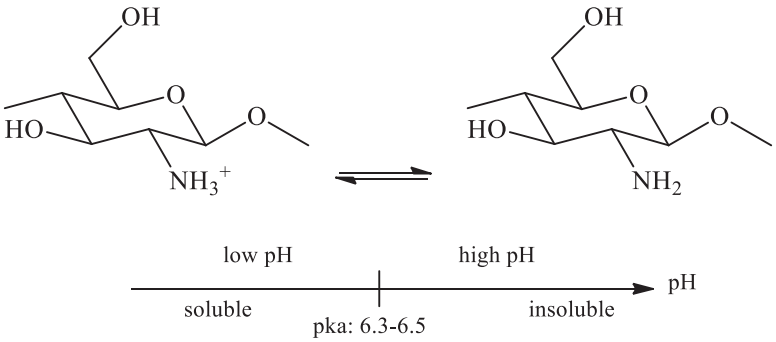

Fig. 2.6 Schematic illustration of chitosan's versatility in aqueous solution: at low pH, less than pK_a, chitosan's amines are protonated conferring polycationic behavior to macromolecules; at higher pH, above pK_a, chitosan's amines are deprotonated and also reactive (useful for derivatization, substitution, etc.); in the latter case, chitosan can undergo associations that can lead to networks such as gels and films to fibers

protonation, and also the distribution of acetyl groups along the macromolecular chain, the latter depending on the conditions of production of chitosan.

The protonation reaction is interesting because, after dissolution, chitosan can be precipitated into beads, cast into films and membranes, spun into fibers or nano-fibers, and also crosslinked to produce fibers or sponges. The processing of chitosan is easier than that of chitin. However, the stability of the products is lower due to the larger hydrophilic character and the pH sensitivity. For better stability, chitosan may be crosslinked using epoxides or glutaraldehyde. Composites and blends are also produced taking advantage of the polycationic properties of chitosan in acidic conditions.

The polyelectrolyte character of chitosan greatly influences its solution properties. There is an interesting structure-property relationship in chitosan. The excellent previous reviews of Vårum and Smidsrød (2004a), Dash et al. (2011), Younes and Rinaudo (2015), and Sahariah and Másson (2017) can be referred to on these

subjects. As a polyelectrolyte, chitosan is able to form electrostatic interactions useful for example in wastewater treatment and presents film-forming capacity and bioadhesivity, properties used in packaging and edible films. These electrostatic interactions are also applied for preparation of layer-by-layer polyelectrolyte capsules or films. The behavior of chitosan in solution is also important for medical applications. Its characteristic features such as being cationic, hemostatic and insoluble at high pH can be reversed by sulfating the amine groups which makes the macromolecule anionic and water-soluble, with the introduction of anticoagulant properties. The presence of the amino groups allows to explain most of chitosan biological properties (Domard and Domard 2001; Vårum and Smidsrød 2004a; Kumirska et al. 2011). Its mucoadhesion can be explained by the interaction between the negatively charged residues in the mucin – the glycoprotein that composes the mucus – and the amino groups positively charged. The hemostatic activity of chitosan can also be related to its cationic character. Red blood cell membranes charged negatively can interact with the positively charged chitosan chains. Besides, chitin shows less effective haemostatic activity than chitosan, confirming this explanation. The polycationic nature of chitosan also allows explaining chitosan analgesic effects. Other examples can be found in the reviews by Croisier and Jérôme (2013), Younes and Rinaudo (2015), and Sahariah and Másson (2017). The facile derivatization also makes chitosan an ideal candidate for the synthesis of biomaterials with specific functionality.

2.2.7 Biodegradation and Toxicity of Chitosan

Chitosan (CAS No. 9012-76-4) is widely regarded as being a non-toxic, biologically compatible, and eco-friendly product (Roberts 1992b; Illum 1998; Thanou et al. 2001; Kean and Thanou 2010). It is approved for excipients, food and dietary applications, and in weight-loss products in Japan, Korea, Italy and Finland. It has been approved by the US Food Drug Administration for use in wound dressing, bandages and hemostatic agents (Illum 1998; Felt et al. 1998; Singla and Chawla 2001; Kato et al. 2003; Illum and Davis 2004; Kean and Thanou 2010; Badwan et al. 2015).

Enzymatically, chitosan can be degraded by enzymes able to hydrolyze glucosamine linkages (Dash et al. 2011). Chitosan is known to be degraded in vertebrates predominantly by lysozyme and by certain bacterial enzymes in the colon. Given adequate time and appropriate conditions, chitosans, in most cases would degrades sufficiently to be excreted. However, as already discussed, the term chitosan represents a large group of structurally different chemical entities that may show different specific characteristics (degree of acetylation, molecular weight, ash content), and biodegradation and toxicological profiles. In addition, chitosan modifications, e.g. by derivatization or cross-linking reactions, could make it more or less toxic and any residual reactants should be carefully removed.

An interesting discussion on these topics can be found in the comprehensive review by Kean and Thanou (2010). These authors concluded that regulatory

agencies encounter many difficulties in approving all existing chitosan-based products as generally regarded as safe (GRAS) materials. Younes and Rinaudo (2015) pointed out that, for biomedical applications, chitin and chitosan need to be highly purified since residual proteins and pigments can cause side effects. Sadler and Funnell (2018) recently discussed the authorized EU health claim for chitosan in relation to its role in the maintenance of normal blood cholesterol levels. Due to the associated risk of elevated blood cholesterol levels with cardiovascular disease, the development of food and beverage products that can help to maintain healthy blood cholesterol concentrations was potentially beneficial to public health. Their review discusses the approved health claim for chitosan, describes the scientific evidence that substantiates the claim, considers how this claim could be used in practice and the consumer perception of health claims.

2.2.8 Derivatives

As already mentioned, chitin is water-insoluble and chitosan exhibits a limitation in its solubility in water and reactivity. The poor solubility of unmodified chitosan in organic solvents also make its utilization limited. Therefore, many studies have paid attention to modify their chemical structure (Austin 1984; Kienzle-Sterzer et al. 1984; Muzzarelli et al. 1986b; Skjåk-Braek et al. 1989; Roberts 1992b, c; Zhang et al. 1993; Hudson and Smith 1998). Often, the methods of modification are adapted from the cellulose world (Muzzarelli et al. 1986c; Kurita 2001, 2006; Rinaudo 2006; Harish Prashanth and Tharanathan 2007; Mourya and Inamdar 2008; Kim and Venkatesan 2014). Chitosan has three types of reactive functional groups: two hydroxyl groups on C-3 and C-6 in each repeating unit and one amino group on C-2 in each deacetylated unit (Fig. 2.1). These groups allow the conjugation of many substituents resulting in new modified derivatives (Fig. 2.7). The main reactions are quaternization (useful to increase the solubility of chitosan in neutral water), acetylation, reductive amination, acylation, phosphorylation, Schiff's bases, and cross-linking modifications (Austin 1984; Kienzle-Sterzer et al. 1984; Kurita 1986, 1998, 2006; Muzzarelli et al. 1986b, c; Skjåk-Braek et al. 1989; Roberts 1992b, c; Hudson and Smith 1998; Kurita 2001, 2006; Harish Prashanth and Tharanathan 2007; Thakur and Thakur 2014; Wang et al. 2016). The main derivatives are quaternary ammonium chitosan salts, carboxymethyl-chitosans, carboxyalkyl-chitosans, aryl-chitosans, hydroxyalkyl-chitosans, sulfated derivatives, phosphorylated chitosan, succinyl-chitosan, and thiolated chitosans (Sashiwa and Aiba 2004; Jayakumar et al. 2006, 2007; Alves and Mano 2008; Mourya and Inamdar 2008). Two update reviews have been compiled by Argüelles-Monal et al. (2018) and Yu et al. (2018). Argüelles-Monal et al. (2018) discussed the latest advances in methods and strategies of chitosan functionalization such as the click chemistry approach. Table 2.6 shows some chitosan derivatives and their potential applications. Carboxymethyl-chitosans are the most fully explored derivatives of chitosan. Under controlled reaction conditions, many products can be obtained with different selectivity (Fig. 2.8) and degree

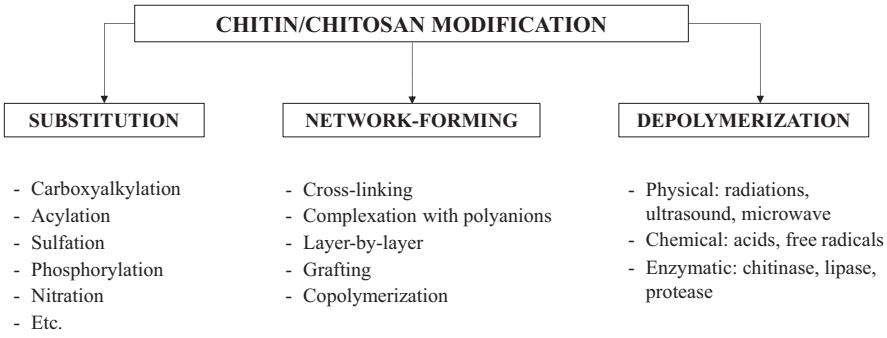

Fig. 2.7 Multifaceted modification potential of chitin and chitosan biopolymers

of substitution. The graft copolymerization of chitosan with particular emphasis on atom transfer radical polymerization as a new strategy to prepare new materials has been discussed by Thakur and Thakur (2014). The authors described the synthesis, characterization, and multifunctional applications of many types of chitosan-based copolymers. They concluded that the technique is promising to incorporate the desired functionalities in chitosan for targeted applications. However, future research needs to look into a better understanding of the structure and chemistry of different polymerization reactions. Wang et al. (2012) also reported that graft copolymerization of chitosan is an alternative route to prepare derivatives for target applications.

Numerous studies have been also conducted to transform the biopolymers chitin and chitosan into low molecular weight chitosans or oligosaccharides using physical, chemical, electrochemical and/or biotechnological processes (Liang et al. 2018; Mourya and Inamdar 2008). Cleavage of glycosidic bonds leads to production of oligomers with important variations in the degree of polymerization (chain lengths). These low molecular weight oligosaccharides, known as chitooligosaccharides, abbreviated COS, or oligochitosans (chitosan oligomers, chitooligomers) present low viscosity and relatively small molecular sizes which in turn make them water soluble and having versatile biological activities such as readily adsorbed *in vivo*, cholesterol lowering, antibacterial and antitumor effects (Hamed et al. 2016; Liaqat and Eltem 2018). They are also soluble in solutions at acid and alkaline pH. Indeed, these products have a remarkably widespread range of biological activities and own an important potential for numerous industrial applications. In target applications such as biomedicine, cosmetics and agriculture, e.g. as antibacterial agent or for plant growth stimulator, low molecular weight chitosans and oligomers are more effective than high-molecular weight chitosans (Peniche et al. 2008; Nwe et al. 2014). Low molecular weight chitosans or oligosaccharides can be obtained from high-molecular weight chitosans by physical, e.g. gamma-ray irradiation, microwave treatment or ultrasonic method, mechanical method (sonication), chemical treatment (oxidation, hydrolysis) using acids or free radicals (HCl, H_2O_2, HNO_2) or enzymatic method using specific or non-specific enzymes, e.g. chitinase, chitosanase, cellulases, lipases, lysozyme, and protease (Liaqat and Eltem 2018; Liang

Table 2.6 Chitosan derivatives and examples of applications

Some chitosan derivatives	Important properties and comment	Application	References
Carboxymethyl-chitosans			
O-carboxymethyl-chitosan N-carboxymethyl-chitosan O,N-carboxymethyl-chitosan N,N-carboxymethyl-chitosan n-Lauryl-carboxymethyl-chitosan	The most fully explored derivatives of chitosan Amphoteric products Water soluble in a wide range of pH The solubility depends on pH Film- and gel-abilities Clarifying agent	Fruit preservation Plant protection Excipients Drug delivery systems Dental care Carriers for hydrophobic cancer drugs Cosmetics Surfactants Beverage industry (clarification) Cotton fabric	Mourya and Inamdar (2008), Elsabee et al. (2009), Jimtaisong and Saewan (2014), Tastan and Baysal (2015), Rocha et al. (2017), Sahariah and Másson (2017), Verlee et al. (2017), and Farion et al. (2018)
Chitosan 6-O-sulfate			
O-sulfated chitosan N-sulfated chitosan 2-N,6-O- sulfated chitosan N-butyl-O-sulfate chitosan N-octyl-O-sulfate chitosan N-palmitoyl-O-sulfate chitosan	Structural analogy to heparin: 2-N,6-O- sulfated chitosan Anticoagulant activity Antimicrobial agent Hemostatic agent Anti HIV-1 activity	Drug delivery Blood anticoagulant Hemagglutination inhibition activity Hemo-compatibility Antitumor activity Antioxidant property Tissue engineering Lipoprotein lipase-releasing activity Treatment of neurological diseases neural repair Superplasticizer	Jayakumar et al. (2007), Elieh-Ali-Komi and Hamblin (2016), Verlee et al. (2017), and Yu et al. (2018)
Quaternized derivatives			
N-substituted quaternary ammonium N,N,N-trimethyl-chitosan chloride N-(2-hydroxyl) propyl-3-trimethylammonium chitosan chloride	Cationic derivatives Water soluble in a wide range of pH Interactions with negatively species Mucoadhesion Better moisture retention and absorption compared to chitosan	Flocculating agent Use in papermaking, paper packaging Antistatic agent Antifungal agent Dental care Drug delivery, gene delivery Gene transfection Interactions with cell membranes	Mourya and Inamdar (2008), Kedjarune-Leggat and Leggat (2011), Huang et al. (2014), Muñoz-Bonilla et al. (2014), LogithKumar et al. (2016), Pardeshi and Belgamwar (2016), and Sahariah and Másson (2017)

(continued)

Table 2.6 (continued)

Some chitosan derivatives	Important properties and comment	Application	References
Alkylated derivatives			
Hydroxyethyl chitosan Hydroxypropyl chitosan Hydroxybutyl chitosan Alkyl-glycosides branched chitosan N-acyl derivatives N-carboxyacyl chitosan N-lauryl derivatives	Amphiphilic products Hydrophobic properties Highly substituted derivatives Modifications require multiple protection and deprotection steps Ability to form physical gels, sponges Rapid gelation kinetics Micelle formation High moisture absorption rate Compatible with neutral and cationic surfactants Rheological modifiers	Surfactant agents Papermaking, hydrophobization of cellulose fibers Hemostatic agent Gene transfection Paints Oil recovery	Macquarie and Hardy (2005), Prabaharan and Mano (2005), Mourya and Inamdar (2008), Prabaharan and Tiwari (2011), Canh et al. (2004), Muñoz-Bonilla et al. (2014), Verlee et al. (2017), and Yu et al. (2018)
N-methylene phosphonic chitosans			
Grafted with alkyl chains Bifunctional derivatives	Anionic products with possible amphoteric character Soluble products Complexing properties Film-forming	Chelating agent Ability for metal removal Cosmetology Anti-corrosion protection	Ramos et al. (2003), Canh et al. (2004), Prabaharan and Tiwari (2011), and Yu et al. (2018)
Target derivatives/reactions			
Schiff's base formation Reductive amination Amide formation Michael addition EDTA-chitosan Thiolated chitosan Thiourea derivatives Phosphorylated chitosan Azidated derivatives Folate-based derivatives Imidazole chitosan Phthaloylated chitosan Conjugated with lipids	Highly porous structure High solubility over a wide range of pH pH-sensitive High transfection efficiency High cellular uptake Mucoadhesive materials	Gene carrier Anticancer agent Drug delivery Ocular drug delivery Delivery of contrast agents Metal chelation	Canh et al. (2004), Macquarie and Hardy (2005), Prabaharan and Mano (2005), Jayakumar et al. (2006, 2008), Mourya and Inamdar (2008), Muñoz-Bonilla et al. (2014), Ahmed and Ikram (2016), Choi et al. (2016), Osman and Arof (2017), and Yu et al. (2018)

(continued)

Table 2.6 (continued)

Some chitosan derivatives	Important properties and comment	Application	References
Carbohydrate-branched chitosans			
Sugar derivatives Glycol-based chitosan Glucosamine chitosan Galactosylated chitosan Mannose or lactose derivatives Cyclodextrin-linked chitosan Cyclic-host bound chitosan	Water-soluble derivatives at wide range of pH Ability to form physical or chemical gels Faster biodegradability Enhanced antimicrobial properties	Drug delivery Self-assembled systems Bone healing Coagulating/ Flocculating agents Adsorbents	Sashiwa and Aiba (2004), Jalal Zohuriaan-Mehr (2005), Prabaharan and Mano (2006), Alves and Mano (2008), Mourya and Inamdar (2008), Prabaharan and Tiwari (2011), Wang et al. (2012), and Muñoz-Bonilla et al. (2014)
Chitosan-grafted copolymers			
Polyethylene glycol-grafted chitosan Polypeptide-grafted chitosans Polyethyleneimine-grafted chitosan Chitosan functionalized poly(vinyl alcohol) Amino acid modification Caproic acid grafted chitosan Stearic acid grafted oligosaccharides Copolymer with acrylamide Copolymer with methylmethacrylate Chitosan-dendrimer hybrid	Water-soluble derivatives The solubility depends on the degree of grafting Induced hydrophilicity or hydrophobicity Biodegradability and non-toxicity: poly(γ-glutamic acid)-chitosan complexes Enhance cell penetrating Improved intracellular release Thermos-responsive gels	Drug delivery Self-assembled systems Oral delivery of insulin Protein vaccine delivery Gene carriers Wastewater treatment	Canh et al. (2004), Sashiwa and Aiba (2004), Harish Prashanth and Tharanathan (2007), Mourya and Inamdar (2008), Wang et al. (2012), Thakur and Thakur (2014), Choi et al. (2016), Rafique et al. (2016), Sahariah and Másson (2017), Farion et al. (2018), Rahangdale and Kumar (2018), and Yu et al. (2018)

(continued)

Table 2.6 (continued)

Some chitosan derivatives	Important properties and comment	Application	References
Cross-linked chitosans			
Gels/Hydrogels Glutaraldehyde chitosan Self-assembled systems Ionic modification Tripolyphosphate as cross-linker Genipin-cross-linked products Modification by aldehydes Glycosaminoglycan-based systems	Biocompatible and cationic products Thermosensitive properties pH-dependent Biosorbents Scaffolds Injectable hydrogels	Wastewater treatment Enzyme immobilization Drug delivery Tissue engineering Veterinary applications Drug eluting stents	Crini (2005), Crini and Badot (2008), Dash et al. (2011), Luna-Bárcenas et al. (2011), Van Vlierberghe et al. (2011), Yao et al. (2012), Xiao et al. (2012), Zhao (2012), Croisier and Jérôme (2013), Zambito (2013), Ahmadi et al. (2015), Tonda-Turo et al. (2016), Ahmed and Ikram (2017), Aminabhavi and Dharupaneedi (2017), and Ali and Ahmed (2018)
Composites/Miscellaneous			
Chitosan complexes Metal ion chelates Chitosan/gelatin microspheres Glycol chitosan Cyanoethyl chitosan Linoleic acid chitosan complex Imidazole chitosan Chitosan-coated materials Hyaluronic acid-chitosan nanoparticles Alginate-chitosan systems Blending with starch Calcium phosphate-based systems Hydroxypropyl-chitosan/gelatin systems Cellulose-chitosan blends Hydroxyapatite composites Modified ceramic nanoparticles Chitosan and clays	Microcapsules Bioactive nanoparticles Nanoemulsions Membranes Delivery vehicles Hybrid scaffolds, multifunctional scaffolds Nanofibers Biosensors	Insulating papers and special papers Dialysis Drug delivery Wound dressing Bone repair Bone tissue engineering Surgical dressing Gene transfection ability Gene therapy Anticancer agent Glucose sensor Antimicrobial textiles Veterinary vaccines Bioimaging agents Wastewater treatment	Mourya and Inamdar (2008), Van Vlierberghe et al. (2011), Dash et al. (2011), Gerdts et al. (2013), Elsabee and Abdou (2013), Islam et al. (2013), Muñoz-Bonilla et al. (2014), van den Broek et al. (2015), Abdul Khalil et al. (2016), Ahmed and Ikram (2016), and Elieh-Ali-Komi and Hamblin (2016)

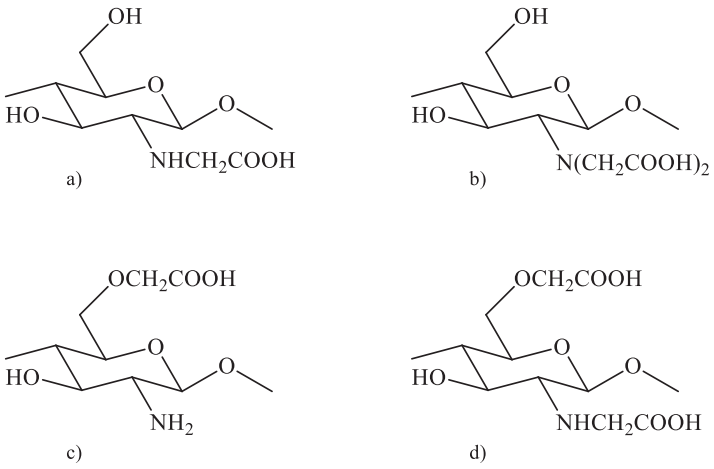

Fig. 2.8 Different chemical structures of carboxymethyl chitosans: (**a**) N-carboxymethyl chitosan; (**b**) N,N-carboxymethyl chitosan; (**c**) O-carboxymethyl chitosan; and (**d**) N,O-carboxymethyl chitosan

et al. 2018). The biotechnological processes have also been gained interest because they permit the production of high-value commercial by-products such as chitinases, lactic acid, antioxidants, valuable protein hydrolysates, carotenoids, and solubilized calcium ions (Philibert et al. 2017).

2.3 Chitosan Applications

Currently, chitosan and its derivatives have practical applications in the form of solutions, suspensions, particles, e.g. beads, resins, spheres, nanoparticles and sponges, gels/hydrogels, foams, membranes and films, fibers, microscopic threads, and scaffolds in many fields: medicine and biomedicine, pharmacy, cosmetology, hygiene and personal care, food industry and nutrition, agriculture and agrochemistry, textile and paper industries, edible film industry and packaging, biotechnology, chemistry, and catalysis, chromatography, beverage industry and enology, photography and other emerging fields such as nutraceuticals, functional textiles and cosmeto-textiles, cosmeceuticals, nanotechnology, and aquaculture (Sandford 1989; Onsoyen and Skaugrud 1990; No and Meyers 1995; Li et al. 1997; Ravi Kumar 2000; Khor 2001; Struszczyk 2002; Honarkar and Barikani 2009; Crini et al. 2009; Ferguson and O'Neill 2011; Kardas et al. 2012; Khor and Wan 2014; Hamed et al. 2016; Amber Jennings and Bumgardner 2017a; Arfin 2017; Badawy and Rabea 2017; Dima et al. 2017; Philibert et al. 2017). Chitosan as biopolymer is also proposed for environmental purposes and applied in clarification and water

purification, wastewater treatment, remediation, sludge dewatering and membrane filtration (Crini and Badot 2008; Bhatnagar and Sillanpää 2009; Sudha 2011; Ravichandran and Rajesh 2013; Yong et al. 2015; Barbusinski et al. 2016; Kos 2016; Nechita 2017; Desbrières and Guibal 2018; Pakdel and Peighambardoust 2018).

Research on chitosan is also very active in electrochemical sensors, bio-imaging, bio-catalysis, ionic liquids, green solvents, adhesives, and detergents. However, the main markets are the food industry and nutrition, and the pharmaceutical, cosmetic, and medicine industries. It is interesting to note that the vast majority of these biological and chemical applications are based on the cationic nature of chitosan and on its versatility as biomaterial. The markets for chitin and chitosan are Japan, USA, Korea, China, Canada, Norway, Australia, France, UK, Poland, and Germany (Ferraro et al. 2010; Nwe et al. 2011b; Badawy and Rabea 2017; Bonecco et al. 2017). Japan dominated the industry accounting for 35% of the market in 2015. Indeed, since the 1980s, Japan is considerably advanced in the technology, commercialization and use of these biopolymers (Badawy and Rabea 2017; Bonecco et al. 2017). The market in this country absorbs about 700–800 tons chitosan per year.

2.3.1 Food Industry

Table 2.7 describes applications of chitosan and its oligosaccharides in food industry. Chitosan has been approved by the US Food and Drug Administration as a generally recognized as sage food additive, dietary fibre (hypocholesterolemic effect), and functional ingredients for the consumer. Chitosan is also approved as a food additive in Japan and in Korea since the 1990s (Vidanarachchi et al. 2011; Gutiérrez 2017). Due to its bioactive nature and cationic character, chitosan is used as nutritional ingredient (food additives, functional food), antimicrobial and antioxidant agent (food protection), for antimicrobial coatings for fruits and vegetables, in anticholesterolemic dietary products, and as nutraceuticals (Shahidi et al. 1999; Agulló et al. 2003; Synowiecki and Al-Khateeb 2003; Vidanarachchi et al. 2011; Kardas et al. 2012; Je and Kim 2012, 2013; van den Broek et al. 2015; Gutiérrez 2017; Han et al. 2018).

Friedman and Juneja (2010) pointed out the outstanding antimicrobial activities of chitosan in solution, powders, and edible films and coating against microorganisms. The better results were obtained with low molecular weight chitosan. Actually, research is directed to new chitosan derivatives and oligomers that can be applied as antimicrobial agent against food microorganisms. These derivatives seem promising, in particular for nutraceutical applications. Kardas et al. (2012) demonstrated that chitosan and its derivatives offer a wide range of unique applications in the food industry including preservation of foods from microbial deterioration, shelf-life extension, formation of biodegradable films and food packaging. The products used as a packaging or coating material can be formed into fibers, films, gels, beads, or nanoparticles. Van den Broek et al. (2015), reviewing the development of chitosan

Table 2.7 Applications of chitosan and its oligosaccharides in food industry (selected reviews)

Topic	Form	Applications
Food technology Nutrition Functional foods Food additives Food mimetic Food protection Food preservation Seafood quality preservation Industrial bio-products Nutraceutical ingredients Dietary food additives Dietary fiber Prebiotic ingredient Flavors/Aromas Essential oils Feed additives Immobilization of enzymes Edible film industry Packaging Coating material Food processing wastes Removal of substances	Solution Film Blend Coating Bead	Additives: fining agent, texture controlling agent, natural flavor extender, emulsifying agent, gelling agent, color stabilizer, dye-binding properties, thickener and stabilizer for sauces, flavor extender Improve nutritional quality Bioactivities: antibacterial, antifungal, antioxidants Preservation of foods from microbial deterioration: protective for fruits Filmogenic properties; microbial films (bactericidal, fungicidal); food wrapping Edible films: controlled release of antioxidants, flavors or nutrients; antimicrobial substances controlled release; reduction of oxygen partial pressure; moisture transfer control; temperature control; controlled rate of respiration Diet foods and dietary fibers: not digestible by humans; glucose dialysis; water holding capacity Hypolipidemic and hypocholesterolemic activities: reduction of lipid absorption, bind fats/lipids, reduce cholesterol Astringency: able to precipitate saliva proteins Non-digestible feed ingredients: prebiotics Production of single cell protein Enhancement of the calcium absorption Encapsulation of nutraceuticals Fractionation of agar Biotechnology: immobilization matrix Bioconversion for the production of value-added food products Recovery of waste material from food-processing discards Infant feed ingredient Animal feed additive Undesired substances removal: dyes, suspended solids Water purification
References		
Goosen (1997), Muzzarelli and de Vincenzi (1997), Shahidi et al. (1999), Domard and Domard (2001), Struszczyk (2002), Agulló et al. (2003), Peniche et al. (2003), Roller and Valley (2003), Begin et al. (2004), Cagri et al. (2004), Shahidi (2004), Krajewska (2005), No et al. (2007), Fernandez-Saiz et al. (2010), Rodrigues et al. (2012), Je and Kim (2012, 2013), Alishahi (2012), Coma (2012), Elsabee and Abdou (2013), Muñoz-Bonilla et al. (2014), van den Broek et al. (2015), Zivanovic et al. (2015), Gallo et al. (2016), Hamed et al. (2016), Gutiérrez (2017), Han et al. (2018), Perinelli et al. (2018), and Wang et al. (2018)		

films and blends as packaging material, also reported a similar conclusion. Due to its antimicrobial and film-forming activities, chitosan has been considered as an appropriate alternative as source of food preservative or coating material for replacing the non-biodegradable and non-renewable polymers, and also reducing the extensive application of harmful pesticides in food protection. Indeed, its films have a selective permeability to gasses (Friedman and Juneja 2010; Fernandez-Saiz et al.

2010; Kardas et al. 2012; Elsabee and Abdou 2013; Muñoz-Bonilla et al. 2014; van den Broek et al. 2015). In addition, the chitosan films have shown superior results, good mechanical properties and they have the advantage of being able to incorporate functional substances such as vitamins and as carriers releasing of antimicrobial agents. However, chitosan films in packaging application are highly permeable to water vapor, and due to their hydrophilic character, they also tend to exhibit resistance to fat diffusion and selective gas permeability. A discussion on the different strategies to overcome these drawbacks can be found in the reviews by Kardas et al. (2012), Elsabee and Abdou (2013), van den Broek et al. (2015), and Wang et al. (2018). Blends, composites and multilayer systems containing chitosan have been proposed as films in food packaging fields and seem promising.

The field of food industry and nutrition is the most important user of chitosan (Gutiérrez 2017; Philibert et al. 2017) and the main markets are localized in Asia (Japan, Korea, China), North America, and in Europe. In the USA, the market in food and beverages is estimated to be 2288 metric tons in 2018. The demand for chitosan is growing rapidly, in particular for potential applications in nutraceutical ingredients and feed stocks. Nutraceuticals as an industry emerged in the early 1990's in Asia, in particular in Japan. The nutraceutical industry includes functional foods, dietary supplements and herbal/natural products. Actually, the US market represents the largest nutraceutical market in the world and the Europe (Germany, France, Netherlands, Sweden) the second. China is expected to be the world's largest consumer of nutraceuticals by 2030. Many products available (ChitoClear®, Chitoseen™-F, MicroChitosan NutriCology®, etc.) are marketed as fat reducers and cholesterol-lowering agents, and also as antioxidant agents. For instance, ChitoClear® is noted as a natural, safe and effective weight loss supplement when used in conjunction with a healthy life style (PRIMEX, Iceland). It seems to have a potential in weight management and obesity treatment. However, there is a debate surrounding the effectiveness of chitosan at blocking fat absorption. The nutraceutical properties of chitosan include its antibacterial, anti-inflammatory, antioxidant, anti-carcinogenic, and antiulcer bioactivities, along with its application as a dietary fiber. Chitosan has non-digestibility in the upper gastrointestinal tract, high viscosity, and high water binding properties (Muzzarelli and de Vincenzi 1997; Roller and Valley 2003; Badawy and Rabea 2017). As a dietary fiber, it has ability to lower cholesterol by blocking the absorption of dietary fat and cholesterols. Chitosan and its derivatives have been shown to facilitate weight and body fat loss in the human body thus decreased systolic and diastolic blood pressure (Philibert et al. 2017). Additionally, it significantly increases the excretion of highly atherogenic saturated fatty acids compared with other fibers. As a valuable prebiotic, chitosan can also promote colonic conditions. Gutiérrez (2017) recently reported that chitosan and its derivatives also exert strong antioxidant activity and their effects are similar to those of phenolic antioxidants. Chitosan products also offer benefits as components of animal feeds and this is also a growing market. They permit food processors to recycle protein from biowastes into animal feed. They have beneficial nutritional properties and they can control the release of feed additives in animals.

2.3.2 Beverage Industry

Table 2.8 summarizes the potential uses of chitosan in beverage industry. In wine production, it can be used for clarification, de-acidification, stabilization, elimination of ochratoxin A, enzymes and other undesired substances, e.g. metals and pesticides (Bornet and Teissedre 2011). Chitosan is also used as eco-friendly coagulant for passion fruit clarification (Domingues et al. 2012) and natural flocculant for beer clarification (Gassara et al. 2015). Recently, Rocha et al. (2017) presented an overview of the recent chitosan-based matrices used for clarification, preservation, encapsulation, and active and intelligent packaging of different beverage types, such as alcoholic, dairy-based drinks, and non-alcoholic, including fruit juices, nectars, concentrated fruit juices, tea, coffee, and tisanes. Only the clarification using chitosan of fungal origin (Oneobrett®, Bactiless™) seems to be well implemented in the market.

Table 2.8 Applications of chitosan and its derivatives and oligosaccharides in beverage industry

Topic	Form	Applications
Beverages Functional beverages Enology Beer industry Packaging	Solution Particle Bead	Filtration and clarification of fruit juices and beverages (wines, beer, tea) Natural flocculant: turbidity removal, removal of suspended solids and colloids (polyphenols, proteins, polysaccharides, minerals) Acidity-adjusting agent: de-acidification of fruit juices and beverages Stabilization of beverages (white wines) Preservative in fruit juices, wines and milk Color stabilizer Prevention of oxidation of the wine color Antimicrobial agent: preservation of drinks from microbial deterioration (bacteria, yeasts, moulds) Natural flavor extender Bioactive compounds encapsulation: protection to oxidation, to improve the bioavaibility of probiotics, making of undesirable taste Preservatives and active packaging Complexing agents in wine industry: metal removal Removal of dead yeast, excess tannin, particulates Water purification
References		
Shahidi et al. (1999), Struszczyk (2002), Chatterjee et al. (2004), Bornet and Teissedre (2005, 2011), Rungsardthong et al. (2006), Domingues et al. (2012), Gassara et al. (2015), Tastan and Baysal (2015), and Rocha et al. (2017)		

2.3.3 Pharmacy

For chitosan, pharmaceutical applications started to appear in the late 1980s (Nagai et al. 1984; Felt et al. 1998; Ravi Kumar 2000). In this field, chitosan and its derivatives have been mainly explored as excipients in drug formulations and in drug delivery systems (Badwan et al. 2015). The new approach consisted of replacing potentially toxic compounds by natural products, which rapidly proved to be promising. The pharmaceutical industry rapidly understood the advantages of using chitosan. Although hundreds of papers and patents related to chitosan-based pharmacy have been published since the late 1980s, this sector continues to interest both the scientific community and the industry, mainly in terms of bioactivities. The most important features and advantages of chitosan can be found in the review by Bernkop-Schnürch and Dünnhaupt (2012). Its derivatization also contributed to expansion of application and decrease toxicity.

The main properties used in the pharmaceutical field are: controlled drug release, e.g. anti-inflammatory naproxen, mucoadhesive properties, *in situ* gelling properties, transfection enhancing properties (deoxyribonucleic acid- and small interfering ribonucleic acid ribonucleic acid-based drugs form stable complexes with chitosan), and permeation enhancing properties (Bernkop-Schnürch and Dünnhaupt 2012). Chitosan also exhibits efflux pump inhibitory properties like other polysaccharides. Chitosan and its derivatives may be used as solutions, gels, tablets, capsules, fibers, films and sponges (Ali and Ahmed 2018). Consequently, they may be used in oral, ocular, nasal, vaginal, buccal, parenteral, intravesical, and transdermal administration, and as implants for drug delivery in both implantable and injectable forms. Table 2.9 reports different methods for the preparation of chitosan-based drug delivery systems (Felt et al. 1998; Ravi Kumar 2000; Peniche et al. 2003; Ravi Kumar et al. 2004; Dash et al. 2011; Ali and Ahmed 2018). Akbar and Shakeel (2018) recently discussed the various forms of chitosan materials as drug delivery device and attempted to report the vast literature available on chitosan based systems in drug delivery applications. Hamedi et al. (2018) also summarized the potential applications of chitosan-based hydrogels for pharmaceutical and biomedical uses, particularly with regard to drug delivery in wound dressings.

Drug delivery applications include not only controlled drug release systems, e.g. site-specific antibiotics delivery in the stomach and controlled release of proteins, but also vaccine and gene delivery (Singh et al. 2018). Chitosan is used as safe excipients in oral dosage form over the last two decades (Felt et al. 1998; Ravi Kumar 2000; Ravi Kumar et al. 2004). Chitosan tablet can exhibit a sustained drug release compared to commercial products. Bernkop-Schnürch and Dünnhaupt (2012) reported that tablets are the likely most favorable dosage form since they provide an accurate dosage, are easy to manufacture and handle, and are favored by patients. The buccal route is an alternative choice to deliver drugs. Chitosan is interesting to be used for buccal delivery due to its mucoadhesive bioactivity and absorption enhancement property. Strong permeation enhancing properties are also mentioned.

Table 2.9 Chitosan-based drug delivery systems prepared by different methods

Form	Applications	Drug(s)
Microspheres	Water-in-oil emulsion cross-linking Coacervation/precipitation Spray-drying Ionic gelation Sieving method	Diclofenac, aspirin, 5-fluorouracil
Beads	Coacervation/precipitation	Bovine serum albumin, salbutamol
Nanoparticles	Emulsion-droplet coalescence Coacervation/precipitation Ionic gelation Reverse micellar method	Insulin, cyclosporin A
Gels/Hydrogels	Cross-linking reactions	Caffeine, lidocaine
Tablets	Matrix coating	Diclofenac, salicylic acid
Capsules/Microcapsules	Capsule shell	Insulin
Films	Wet casting from salt solutions	Testosterone, trypsin
Sponges/Foams	Freeze drying Reactions in supercritical fluids	Triamcinolone acetonide

Injectable preparations containing chitosan have received considerable attention within the last years. The properties of chitosan also resulted in the development of vaccine delivery (Bernkop-Schnürch and Dünnhaupt 2012). The transmucosal administration of drugs has been described in detail by Illum and Davis (2004). The transmucosal absorption promoter effect of chitosan is important for nasal and oral delivery of polar drugs to administrate peptides and proteins, and for vaccine delivery. Films and fibers prepared using chitosan and chitin were developed for tissue engineering and wound care dressing, as oral mucoadhesive and water-resisting adhesive by virtue of their release characteristics and adhesion (Kato et al. 2003; Illum and Davis 2004). The chitosan-based fiber production was discussed in detail by Pillai et al. (2009). Promising developments were under way not only in biomedicine but also in emerging domains such as nutraceuticals and cosmeceuticals. Indeed, pharmaceutical formulations containing chitosan and derivatives are also proposed for slimming application, body weight management and as cosmetics to enhance skincare efficacy for example. The use of nanocomposites in drug delivery also seems promising as recently reported by Ali and Ahmed (2018). Other applications of chitosan and its derivatives are described in Table 2.10.

2.3.4 Medicine

The medical and biomedical potential applications of chitosan include pharmaceutical formulation and drug delivery (antibiotics, anti-inflammatory substances, vaccines, proteins, peptides, growth of factors), antimicrobial applications, gene delivery, gene therapy, wounds healing and burns, regenerative medicine, tissue

Table 2.10 Applications of chitosan and its derivatives including oligosaccharides in pharmacy (selected reviews)

Topic	Form	Applications
Excipients Drug delivery Drug release Vaccines Biopharmaceutics Nutraceuticals Body weight management Cosmeceuticals Dermatology Ophthalmology Oral drug delivery Imaging agents	Solution Powder Microsphere Bead Tablet Capsule, microcapsule Nanoparticle Nanocomposite Sponge	Excipients Encapsulation of sensitive drugs: to increase and to modulate drug release rate Controlled drug delivery carriers Controlled release of proteins and peptides Gene delivery (nucleic acid) Dermatological products: to treat acne Hydrating agent Hemostatic and anticoagulant Bacteriostatic agent Mucoadhesion Biological adhesive: water-resisting adhesive *In situ* gelation Transfection Permeation enhancement Healing: wound healing, self-healing Efflux pump inhibitory properties Products for radio-pharmaceutical domains Nutraceutical ingredients
References		
Goosen (1997), Illum (1998), Dodane and Vilivalam (1998), Felt et al. (1998), Ravi Kumar (2000), Hejazi and Amiji (2001), Agnihotri et al. (2004), Canh et al. (2004), Ravi Kumar et al. (2004), Prabaharan and Mano (2005), Varshosaz (2007), Dash et al. (2009), Kang et al. (2009), Hamman (2010), Riva et al. (2011), Bernkop-Schnürch and Dünnhaupt (2012), Xiao et al. (2012), Zhao (2012), Yong and Wong (2013), Badwan et al. (2015), Mateescu et al. (2015b), Majekodunmi (2016), Lucio and Martínez-Ohárriz (2017), Parhi (2017), Ahsan et al. (2018), Akbar and Shakeel (2018), Ali and Ahmed (2018), Krishnaswami et al. (2018), Naskar et al. (2018), and Tripathi and Singh (2018)		

engineering (bone, ligament, cartilage, tendon, liver, neural and skin regeneration), applications in cancer (treatment, therapy, diagnostic strategy), dermatology, ophthalmology, dentistry, biosensors, and many other applications such as bio-imaging (magnetic resonance imaging), support for immobilized enzymes and veterinary medicine (Table 2.11). For medical applications, chitosan and its derivatives as chitooligosaccharides can be easily processed into different forms such as solutions, gels/hydrogels, sponges, microparticles/nanoparticles, membranes and films (pure films or blends, adhesives), and fibers/nanofibers (Allan et al. 1984; Hon 1996; Dumitriu 2001; Yilmaz 2004; Jayakumar et al. 2010b, 2011b; Riva et al. 2011; Elieh-Ali-Komi and Hamblin 2016; Amber Jennings and Bumgardner 2017a, b; Rijal et al. 2017; Ali and Ahmed 2018; Hamedi et al. 2018; Liaqat and Eltem 2018). The use of chitosan-based materials in 2D-scaffolds such as films and fibers and 3D-scaffolds such as gels and sponges is discussed in the reviews by Croisier and

Table 2.11 Applications of chitosan and its oligosaccharides in medicine and biomedicine (selected reviews)

Topic	Forms	Applications
Drug delivery Biomedicine Biomedical engineering Biofabrication Tissue engineering Regenerative medicine Wound dressing Growth factor Biomedical adhesives Medical materials Medical devices Implants Orthopedics Therapeutic domains Microbiology Immunization Immunology Gene therapy Cell biology Cell therapy Cell adhesion and proliferation Cell-biomaterials interactions Protein adsorption onto biomaterials Cancer diagnosis Cancer therapy Carriers of anticancer drugs Ophthalmology Bio-sensing Bio-imaging Bio-printing 3D-printing	Solution Gel/hydrogel Powder Microsphere Microcapsule Bead Nanoparticle Film Fiber, nanofiber Nonwoven bioactive fiber Sponge 2D- and 3D-scaffolds Shaped object Adhesive	Drug delivery: delivery of antibiotics, peptides, proteins, vaccines Growth factor delivery Biological response modifier Gene delivery, targeted delivery, deoxyribonucleic acids therapy, gene therapeutics, small interfering ribonucleic acid delivery Antifungal, antimicrobial, anti-infectious Hemostatic effects; enhances blood coagulation Anticoagulant hydrogel containing heparin Blood cholesterol control Adjuvant properties Bioadhesive Artificial skin; skin burn Promotes tissue growth; tissue repair and regeneration Cartilage tissue engineering Scaffolds for cell culture; stimulates cell proliferation Material supporting nerve repair Wound healing properties Scaffolds for bone regeneration: bone substitutes and cements; rebuilding of bone Biocompatible and biodegradable materials for use as implants, blood substitutes, blood vessels or wound dressing material Antitumor agent, tumor inhibition Treatment of leukemia, diabetes Sutures, surgical threads, bandages, sponges Dental implants Contact lenses Magnetic resonance imaging
References		

(continued)

Table 2.11 (continued)

Topic	Forms	Applications
Francis Suh and Matthew (2000), Khor and Lim (2003), Elder et al. (2004), Berger et al. (2004a, b), Illum and Davis (2004), Sashiwa and Aiba (2004), Barbosa et al. (2005), Yi et al. (2005), Shi et al. (2006), Jayakumar et al. (2007), Lee (2007), Alves and Mano (2008), Kang et al. (2009), Muzzarelli (2009, 2011), Venkatesan and Kim (2010), Khor (2011), Sahoo and Nayak (2011), Dash et al. (2011), Dutta et al. (2011), Jayakumar et al. (2011a), Lakshmanan et al. (2011), Liu et al. (2011), Luna-Bárcenas et al. (2011), Sarmento and das Neves (2012), Wang et al. (2012), Croisier and Jérôme (2013), Kim and Pangestuti (2013), Anitha et al. (2014), Jana et al. (2014), Junginer and Sadeghi (2014), Muñoz-Bonilla et al. (2014), Balan and Verestiuc (2014), Azuma et al. (2015), Pokhrel et al. (2015), Dutta (2016), Ahmed and Ikram (2016), Elieh-Ali-Komi and Hamblin (2016), Choi et al. (2016), LogithKumar et al. (2016), Ahmad et al. (2017a, b), Amber Jennings and Bumgardner (2017b), Balagangadharan et al. (2017), Bano et al. (2017), Ellis and Korbutt (2017), Harris et al. (2017), Layek and Singh (2017), Vunain et al. (2017), Aljohani et al. (2018), Ahsan et al. (2018), Ahmed et al. (2018), Ahsan et al. (2018), Ali and Ahmed (2018), Anraku et al. (2018), Baranwal et al. (2018), De Mori et al. (2018), Dimassi et al. (2018), Ding et al. (2018), Hamedi et al. (2018), Hu et al. (2018), Li et al. (2018), Komi et al. (2018), Krishnaswami et al. (2018), Liaqat and Eltem (2018), Mohandas et al. (2018), Nezakati et al. (2018), Oryan et al. (2018), Pellá et al. (2018), Qasim et al. (2018), Shariatinia and Jalali (2018), Singh et al. (2018), Tripathi and Singh (2018), Xing et al. (2018), Vasconcelos and Pomin (2018), Xu et al. (2018), and Zhao et al. (2018)		

Jérôme (2013), Anitha et al. (2014), Ahmed and Ikram (2016), and LogithKumar et al. (2016), with a special focus on tissue engineering and wound healing applications. Their bacteriostatic and fungistatic properties are particularly useful for wound treatment. This application combines two of the most interesting properties of chitosan: antimicrobial activity and biocompatibility. Chitosan and its oligosaccharides have also a stimulatory effect on cells. Materials in the forms of nonwovens, nanofibers, composites, films and sponges can accelerate wound healing and dermal regeneration. These products have been on the market since the early 1990s, mainly in North America and Asia, and more recently in Europe (Germany, France). Actually, the main biomedical commercial applications of chitosan are in wound healing.

Various forms of wound dressing chitosan-based materials are commercially available in the market: HemCon® Bandage, ChitoGauze® PRO, ChitoFlex® PRO, ChitoSam™,Syvek-Patch®, Chitopack C® and Chitopack S®, Chitodine®, ChitosanSkin®, TraumaStat®, TraumaDEX®, Celox™, etc. For example, HemCon® Bandage is an engineered chitosan acetate preparation designed as a high-performance hemostatic dressing (HemCon Medical Technologies, Inc., USA). Other biomedical products are in the market. Reaxon® (Medovent, Germany) is a chitosan-based nerve conduit which is resistant to collapse and helps to avoid the undesired drawbacks of autografts. This hydrogel is bioactive (supports nerve regeneration equivalent to the autograft), biocompatible (prevents irritation and inflammation), antiadhesive (inhibits scar tissue and neuroma formation), and antibacterial (prevents infection). Its particular structure also facilitates the transport of

nutrients and oxygen. ChitoSeat™ is a family of chitosan based hemostatic sealants that are suitable for surgical hemorrhage of hard and soft tissue (LUNA, USA).

In tissue engineering, the aim is to restore or replace damaged body parts or lost organs by transplanting supportive scaffolds with appropriate cells that in combination with biomolecules generate new tissue (Dash et al. 2011; Saravanan et al. 2013; Ahmed et al. 2018). Dash et al. (2011) reported that chitosan is an ideal dressing in wound-healing applications due to the fact that not only it protects the wound from bacterial infection but also it promotes healing and it produces less scarring. In addition to the reparative nature of the material, it can also deliver a therapeutic payload to the local wound, for example, fibroblast growth factor-2 which stimulates angiogenesis by activating capillary endothelial cells and fibroblasts. The potential use of chitosan in this topic is, however, limited due to is poor solubility in water, faster *in vivo* depolymerization/degradation, hemo-incompatibility, and also weak antimicrobial property. To overcome these problems, chitosan derivatives have been proposed as novel scaffold materials for tissue engineering. A discussion on the use of many derivatives in bone tissue engineering as emerging products can be found in the recent reviews by LogithKumar et al. (2016) and by Ahmed et al. (2018).

For bone regeneration, several injectable materials were used. Chitosan-calcium phosphate composites appear to have a promising clinical application. Chemically modified hyaluronic acid-chitin and chitosan-hyaluronic acid material were reported to be osteoinductive and exhibited rapid degradation and neovascularization in vivo. Chitosan scaffolds are potentially a useful alternative to synthetic cell scaffolds also for cartilage tissue engineering. Anitha et al. (2014) reviewed the use of chitosan-based membranes and scaffolds not only for tissue engineering and wound healing but also as anticancer drug delivery, osteogenic drug delivery, and growth factor delivery. The key features of these biomaterials are their biodegradability, cytocompatibility, multi-functionality and specific mechanical properties. n-Lauryl-carboxymethylcellulose has been proposed as carrier for hydrophobic cancer drugs. This amphiphilic substance is safe in terms of membrane toxicity. Materials for cancer chemotherapy are important but still under development (Saneja et al. 2016). Chitosan-based biomaterials were proposed against diabetes and related complications (Kim and Karadeniz 2013; Kim and Pangestuti 2013; Karadeniz and Kim 2014a, b) and also as new adhesives (Mati-Baouche et al. 2014).

Chitosan is a biocompatible substance with no antigenic properties, and thus it is perfectly compatible with living tissues. Its hemostatic and antithrombogenic bioactivities make it suitable in all fields of medicine: for controlled drug release, drugs encapsulation, enzymes and cells immobilization, and also as gene carriers. Chitosan is biodegradable and enzymes break it down into oligo-products that are then dealt with by the metabolism. Many chitosan derivatives are also biocompatible and non-toxic with living tissues. Other advantages of chitosan-based materials often cited are related to their hydrophilic property, biodegradability, antibacterial activity, bioadhesivity, mucoadhesivity, and complexing property. Like alginate polysaccharide, chitosan has the characteristic of forming gels in addition to possessing viscosity-related properties, complete biodegradability, and even anti-tumor influence. Chitosan forms films that are permeable to air. It facilitates cellular regeneration

while protecting tissues from microbial attacks. Chitosan has also a stimulating effect on the regeneration of tissues. It is used in making an artificial skin for skin grafts on high degree burns and in surgical applications (suture threads). Chitosan can trap lipids at their insolubilisation pH in the digestive tract. It considerably reduces the level of cholesterol in the blood. Chitosan possesses bioadhesive properties which make it of interest in adhesive sustained release formulation required. Mucoadhesivity permit to enhance the adsorption of drugs especially at neutral pH (Dash et al. 2011; Liu et al. 2011; Dutta 2016; Amber Jennings and Bumgardner 2017a, b; Vunain et al. 2017).

Genetic materials such as deoxyribonucleic acids and ribonucleic acid are used in gene therapy as pharmaceutical agents to treat various diseases (Lee 2007; Dash et al. 2011; Kedjarune-Leggat and Leggat 2011; Choi et al. 2016; Badawy and Rabea 2017). However, the use of genetic materials is limited due to rapid degradation by nuclease, large size, poor cellular uptake, high anionic charge density, and also non-specificity. To overcome these problems, non-viral vectors as cationic chitosan were proposed as promising delivery biomaterials in gene therapy (Kedjarune-Leggat and Leggat 2011). Chitosan is a prominent system-based gene delivery vector due to its facility to form complexes, biodegradability and biocompatibility, although its transfection efficiency and cell specificity are low. Its role in gene delivery is supported by its ability to protonate in acidic media forming a complex with deoxyribonucleic acids through electrostatic interactions (Choi et al. 2016). The chitosan-deoxyribonucleic acids complexes are easy to prepare and are more effective compared to the commonly used systems. Chitosan/deoxyribonucleic acids complexes were reported to transfect into various cell types: human embryonic kidney cells, cervical cancer cells, primary chondrocytes, and fibroblast cells. As recently reviewed by Badawy and Rabea (2017), the transfection efficiency of chitosan depends on the degree of acetylation, molecular weight, pH of the transfecting media, cell type, and the charge ratio between the luciferase plasmid to chitosan. Limitations in the use of chitosan for non-viral gene therapy were previously reported by Kedjarune-Leggat and Leggat (2011). Most of the results on gene therapy using chitosan were obtained from experiments in vitro and further research is needed in vivo. In addition, studies still need to understand the effects of the characteristics of the carriers on cellular entry and intracellular trafficking processes (Choi et al. 2016).

Sulfated chitosan has the ability to interfere with blood clotting process. This is a subject of extensive medical applications. Compared to heparin, this derivative has been shown to possess high anticoagulant potency. Unlike heparin, sulfated chitosan does not show anti-platelet activity, which causes excessive bleeding in patients (Badawy and Rabea 2017). Another example of extensive medical studies concern the production of chitosan-based vaginal tablets used as drug delivery systems due to their adequate release properties and good adhesive properties (El-Kamel et al. 2002; Raafat and Sahl 2009; Bernkop-Schnürch and Dünnhaupt 2012). However, as claimed by Raafat and Sahl (2009), the antimicrobial properties of chitosan might have a negative impact on the vaginal microflora. Its vaginal use for treatment of chronic diseases has therefore to be seen with caution. Another application related

to chitosan is based on the fact its modified particles provide an excellent template for bio-imaging. Chitosan-based nanoparticles containing imaging agents were studied for radiopharmaceutical applications and magnetic resonance imaging (Kumar et al. 2004; Dash et al. 2011).

The medical and biomedical potential applications of chitosan also include ophthalmology, dentistry, and veterinary medicine. In ophthalmology, due to their non-toxic character and permeation enhancing properties, chitosan-based formulations are used as drug delivery systems including coated colloidal systems, hydrogels, and nanoparticles (Bernkop-Schnürch and Dünnhaupt 2012; Elieh-Ali-Komi and Hamblin 2016; Krishnaswami et al. 2018). Chitosan possesses all the characteristics required for making an ideal contact lens: optical clarity, mechanical stability, optical correction, gas (oxygen) permeability, wettability and immunological compatibility (Elieh-Ali-Komi and Hamblin 2016; Badawy and Rabea 2017). The antimicrobial activity, film-forming ability, and wound-healing properties also make chitosan suitable for development of ocular bandage-lenses for traumatic injuries (Elieh-Ali-Komi and Hamblin 2016).

2.3.5 Dentistry

The relevant properties of chitosan cited for dentistry are: bioactivity, anti-inflammatory, wound healing, hemostatic activities and bone repair (Queiroz et al. 2015; Kmiec et al. 2017; Navarro-Suarez et al. 2018; Zheng et al. 2018). Chitosan is used in the form of solution (acetic or methanesulfonic acid used as solvents), microspheres, hydrogel and toothpastes, and its association with additives such as amorphous calcium phosphate, amelogenin and quinic acid improved the ability of these chitosan preparations in preventing dental caries and enamel erosion. Its applications in dentistry are described in Table 2.12. Chitosan in gel/hydrogel form applies to the treatment of chronic periodontitis and canker sores. Due to its inherent versatility, efficiency and ability to act as a protective barrier to the penetration of acids into enamel and its mineral loss, chitosan can play an important role in preventive dentistry. In addition, when associated with remineralizing agents, chitosan preparations are able to repair early caries lesions (Queiroz et al. 2015). Toothpastes, mouthwashes and chewing gums based on chitosan and herbs fullness functions antimicrobial effect on oral bio film and reduction of the number of *Streptococcus mutans* in the oral cavity (Kmiec et al. 2017). They freshen the breath and prevent the formulation of plaque and tooth decay. Salts of chitosan added to tooth paste mask the unpleasant taste of silicon oxide and bind powders, so that they maintain their granular shapes. Chitosan complex and fluoride microparticles increase fluoride absorption and protection cavities. Endodontic cements based on chitosan reduces inflammation and support bone regeneration. Navarro-Suarez et al. (2018) recently discussed the use of nanotechnology in dentistry and the latest innovations in nanobiomaterials products. Zheng et al. (2018) also reviewed the application of biomaterials in dentistry, with a focus on new techniques using a

Table 2.12 Applications of chitosan and its oligosaccharides in dentistry

Topic	Form	Applications
Dental surgery	Solution	Agent to prevent diseases: dental caries,
Dental therapy	Hydrogel	periodontitis, erosive lesion, dental plaque inhibitor
Dental materials	Toothpastes	Ingredient in dentifrices
Implants	Bioadhesive	Oral care, dental care: toothpaste, chewing gum
Drug delivery systems	Powder	Chitosan-containing chewing gum having
Oral hygiene	Granule	antibacterial effects or to increase salivary secretion
Antibacterial effect	Nanoparticle	Delivery of fluoride
Restorative dentistry	Sponge	Dental adhesives
Dental composites	Composites	Nanobiomaterials
Wound healing		Agent to promote wound healing in bone tissue
Nanodentistry		Scaffolds and carriers for molecular therapy
		Cell protective activity
References		
Sapelli et al. (1986), Khor and Lim (2003), Stamford Arnaud et al. (2010), Hayashi (2011), Keegan et al. (2012), Hayashi et al. (2013), Farea et al. (2014), Queiroz et al. (2015), Kmiec et al. (2017), Wieckiewic et al. (2017), Elkassas and Arafa (2017), Ahsan et al. (2018), Navarro-Suarez et al. (2018), and Zheng et al. (2018)		

combination of scaffolds, cells and biologically active molecules to assemble functional constructs that restore, maintain or improve damaged tissues for dental purposes.

2.3.6 Veterinary Medicine

Senel and McClure (2004) previously reviewed the applications of chitosan in veterinary medicine including wound healing, bone regeneration, analgesic and antimicrobial effects. They also discussed the potential application of chitosan to drug and vaccine delivery in veterinary species, and as nutritional ingredient. Given the restrictions imposed by financial and animal restraint considerations, especially in fanning applications, the veterinary drug delivery areas most likely to benefit from chitosan are the delivery of chemotherapeutics such as antibiotics, antiparasitics, anaesthetics, painkillers and growth promotants to mucosal epithelium for absorption for local or systemic activity, and the delivery of immunomodulatory agents to the mucosal associated lymphoid tissue for induction or modulation of local immune responses. Other applications in veterinary medicine are showed in Table 2.13. The use of nanoparticles for drug delivery, diagnostics and vaccine formulation has described by Underwood and van Eps (2012). Nanomedicine using innovative nanosystems seems to be a promising domain. Products have already reached the market. For instance, chitosan-based nutritional supplements (Epakitin™, Nutri+Gen®) are commercially available for use as essential nutrients supplement or in chronic kidney disease in dogs and cats. The Epakitin formulation containing both chitosan and calcium carbonate is a safe and highly palatable kidney-protective

Table 2.13 Applications of chitosan and its oligosaccharides in veterinary medicine

Topic	Forms	Applications
Delivery systems	Solution	Time release drugs for animals
Vaccine delivery	Powder	Mucosal formulations
Adjuvant	Microsphere	Mucosal delivery of antigens
Biological properties	Microcapsule	Improve the immune response
Mucosal immunization	Nanoparticle	Hemostatic
Wound healing	Gel, hydrogel	Wound-healing activity
Tissue regeneration	Sponge	Regenerative medicine: tissue engineering
Nutritional supplement	Film	Vaccines
Nanomedicine	Filament	Surgical threads
		Food for dogs
		Reduce urea levels
		Body-care products: shampoo, sprays
References		
Şenel and McClure (2004), Şenel (2011), Underwood and van Eps (2012), Drewnowska et al. (2013), Gerdts et al. (2013), Tonda-Turo et al. (2016)		

phosphate binder. Many body care are also available (ChitoCure®, ChitoClear®): shampoo, ear-cleaner, conditioner, sprays for companion animals, etc.

2.3.7 Cosmetics

It is possible to produce chitosans as well as chitosan derivatives with varying chain lengths and differentiated properties for applications in cosmetics, hygiene and personal care. The molecular weight of most chitosan products are so high that they cannot penetrate the skin and this is an important advantage that make it suitable for skin care. These materials include chitosan hydrochloride, chitosan acetate, chitosan lactate, carboxymethyl-chitosan, quaternized-derivatives, oligosaccharides, and also chitin sulphate and carboxymethyl-chitin. They can be dissolved in aqueous solutions or used in solid form. In cosmetics, the specific properties employed are: cationic (chitosan and hair carry opposite electrical charges), bacteriostatic, fungistatic, antistatic, film-forming, moisture-retaining (chitosan retain moisture in low humidity and maintain hair's style in high humidity), and controlled release of bioactive agents. Chitosan is also of great interest in cosmetic formulations because it is compatible with other ingredients such as starch, glucose, saccharose, polyols, oils, fats, waxes, acids, non-ionic emulsifiers and non-ionic water-soluble gums. However, chitosan is incompatible with ionic gums, sulphonated surface-active agents, alkalis, and sulphuric acids. Chitosan and its derivatives can be combined with other hydrating agents, solar filters and other bioactive products used in the formulations. They facilitate their effects. Some derivatives of chitosan can form foam and create emulsifying actions.

Some of the chitosan applications (Table 2.14) are: hair care, e.g. shampoos, coloring products, hairspray, and setting lotion, creams and lotions (face, hand and

Table 2.14 Applications of chitosan and its oligosaccharides in cosmetics

Topic	Form	Applications
Toiletry Hygiene Personal care Skin care Oral care Dental care Hair care Cosmeceuticals Fragrances Essential oils	Solution Powder Film	Functional additives Moisturizers: maintain skin moisture, tone skin Thickening agent Hydrating and film-forming agent Role in surfactant stability; stabilize emulsion Antistatic effect Bacteriostatic Encapsulating agent Delivery systems Products: shampoos, creams, skin creams, creams for acne treatment, lotions, bath lotions, nail polish, fixtures, make-up powder, lacquers, nail lacquers, nail enamel, varnishes, hair sprays, hair colorants, wave agents Cleaning products: cleansing milk, face peel, facial toner, soap, bath agent Hair care: elastic film on hair, increase its softness and mechanical strength, improve suppleness of hair, remove oils and sebum from hairs; reduce static electricity in hair, retain moisture and maintain hair's style Oral care, dental care: toothpaste, chewing gum
References		
Goosen (1997), Struszczyk (2002), Rinaudo (2006), Crini et al. (2009), Muñoz et al. (2012), Lima et al. (2012), Senevirathne et al. (2012), Chalongsuk and Sribundit (2013), Jimtaisong and Saewan (2014), Costa and Santos (2017), and Rahangdale and Kumar (2018)		

body products), color cosmetics (make-up, nail polish, eye shadow, and lipstick), deodorizing products, micro-encapsulation of active agents, and dental care, e.g. toothpaste, tooth-gel, and mouth wash. Chitin is also interesting in cosmetology because it is well tolerated by the skin. It is an effective hydrating agent and a film-forming tensor having two advantages often cited: it supplies water and it avoids dehydration. Chitosan and chitin also present chelating properties towards metals that are responsible for very many contact allergies. Carboxymethyl-chitosans products are mainly used in cosmetics as antioxidant agent, moisture absorption-retention agent, antimicrobial agent, delivery system and emulsion stabilization. Jimtaisong and Saewan (2014) comprehensively discussed the utilization of carboxymethyl-chitosans as multifunctional ingredients in the formulation of cosmetics. They also included in their review information on cytotoxicity of these products to ensure their safety.

Numerous chitosan-based products for cosmetic use are commercially available: Curasan™, Hydamer™, Zenvivo™, Ritachitosan®, Chitosan MM222, etc. Cosmetic industry is a strongly growing market. The cosmeceutical market has also been growing particularly rapidly of late. Cosmeceuticals (Chitoseen™-K) are cosmetics with pharmaceutical/medicinal benefits. These products seem to provide not only a health benefit but also cosmetic qualities. They contain essential oils and active ingredients such as vitamins, enzymes, antioxidants, and phytochemicals. They can be applied as creams, lotions and ointments (Lima et al. 2012; Muñoz

et al. 2012; Senevirathne et al. 2012). Since the last decade, they continue to revolutionize the world of hair, lip, tooth and skin care by offering safe and natural ingredients for consumer's personal use.

2.3.8 Agriculture

Applications of chitosan in agriculture are summarized in Table 2.15. Chitosan products are used in plant protection from the 1990s against plant pathogenic bacteria that induce decay and harmful effects of agricultural crops during the growing season and postharvest phase (Yin and Du 2011). They behave as bactericidal (killing the bacteria) and/or bacteriostatic (hindering the growth of bacteria). However, the exact mechanism is still not fully understood. A discussion on models proposed for antibacterial actions of chitosan can be found in the review by Muñoz-Bonilla et al. (2014). The most accepted mechanism involves the polycationic character of chitosan which permits to interact with negatively charged species (bacterium cell membrane). The chelating properties of chitosan also make it an excellent antifungal agent (Rabea et al. 2003; Muñoz-Bonilla et al. 2014; Badawy and Rabea 2016; Divya and Jisha 2018). The presence of chitosan activates many defense responses in plants. Usually, it is employed in plant disease control as a powerful elicitor.

Table 2.15 Applications of chitosan and its oligosaccharides in agriculture

Topic	Form	Applications
Plant protection Antimicrobial agent Antioxidant Horticulture Agrochemistry Soil enrichment Postharvest Edible films	Solution Spray Coating Powder Gel Powder Nanoparticle	Protection of plants Coating material: seeds, fruits, vegetables Stimulation of plant growth and plant production Increase of crop yields Reduce the growth of phytopathogenic fungi Effects on gene expression Pest control Soil treatment (nutrients) Modify plant-microbial interactions Elicitor to stimulate the accumulation of secondary metabolites and to induce plant defenses Frost protection Controlled agrochemical release Fertilizers and biocontrol agent (time release of products) Bio-fertilizer, fertilizer protectant Spray for pesticide removal (fruits) Pesticide formulations; biopesticides; biofungicides
References		
Goosen (1997), El Hadrami et al. (2010), Yin and Du (2011), Sharp (2013), Muñoz-Bonilla et al. (2014), Katiyar et al. (2014), Xing et al. (2015), Badawy and Rabea (2016, 2017), Bautista-Baños et al. (2016), Hadwiger (2017), Ippólito et al. (2017), Orzali et al. (2017), Divya and Jisha (2018), Grande-Tovar et al. (2018), and Sharif et al. (2018)		

Chitosan products were proposed as devices for controlling the release of agrochemicals (fertilizers, pesticides).They are used as biocides either alone or blended with other products against plant diseases (control of plant bacteria and fungi), pests and insects, plant growth promotion, seed-coating, and postharvest (Divya and Jisha 2018; Grande-Tovar et al. 2018; Sharif et al. 2018). Chitosan has also inhibitory effect on viruses and viroid of plants. It has a great potential as biopesticide. It can function as a seed-soaking agent, a root-applying agent, and a spray agent. These activities play an important role on plant disease control and stress resistance. Blending of chitosan with other products, such as gum, starch, and alginate, is a convenient method to improve its properties for slow release of pesticides. The use of chitosan products can elicit defense to more than 60 diseases on several plants. Their potent effect on plant disease control is from their antimicrobial and plant innate immunity elicited activity. The inhibition activity was observed on different stages of fungal growth such as mycelia, sporulation, spore viability and germination, and production of fungal virulence factors. Chitosan products can be used in various ways: coating seed (soybean, cotton, cucumber, wheat, rice), soil enrichment (for potato, soybean lettuce, spinach), foliar spraying (for peanut, soybean, cabbage, rice, maize, cotton), supplement in hydroponic (rice, wheat, peanut), and supplement in plant tissue culture medium (*Chrysanthemum*, *Limonium*, carrot). As seed coating agent, they protect plants, e.g. cotton, tomato, wheat, and have a positive effect on germination rate, seedling growth parameters, and the yield of different cultivars, e.g. soybean sprouts, ornamental plants, maize, wheat, lentil, rice, and peanut. Their bioactivities as antifungal activity, enhancement crop yield, induction of defensive system of plants and plant-growth promotion play key roles in their application for agriculture.

The two main problems of chitosan in agriculture applications are commercial chitosan standardization and solubility. Indeed, chitosan bioactivities are dependent on several parameters including degree of acetylation, molecular weight, concentration of chitosan, pH of the solution, its viscosity, and the target of microorganism. The antimicrobial activity of non-modified chitosan against various microorganisms such as bacteria, yeasts, fungi and viruses has received much attention in the last two decades. The literature data showed that this bioactivity property depends on various factors such as molecular weight, which is probably the main factor affecting the efficacy, although literature is sometimes contradictory. Generally, the antibacterial activity increase as the molecular weight decreases. However, it is difficult to find a clear correlation between the molecular weight and this bioactivity (Badawy and Rabea 2017). In spite of its unique biological aspects, the water-insoluble property is another major limiting factor for its wide application in agriculture. Recently, to overcome these problems, chitosan derivatives and oligomers produced by enzymatic and chemical modifications have been proposed. It is expected that these biopolymers would be promising candidates in agriculture. Research are also in progress on the mechanisms of chitosan-induced defense and on the signal perception of chitosan. Nanotechnology using innovative chitosan nanoparticles seems to be a promising domain. The methods of preparation of

chitosan nanoparticles and their potential applications as antimicrobial agent, plant growth-promoting agent and plant protector have been discussed in the recent review by Divya and Jisha (2018).

2.3.9 Aquaculture

A prerequisite for the greater use of chitin in industry is cheap manufacturing processes and/or the development of profitable processes to recover chitin and byproducts such as proteins and pigments. It is well-known that the recovery of chitinous products from wastes is an additional source of revenue. Crustacean shells contain considerable quantities of carotenoids which so far have not been synthesized, and which are marketed as a fish food additive in aquaculture, mainly for salmon. The use of chitosan and its derivatives in the aquaculture was described by Alishahi and Aïder (2012). It can be used as functional food, nutritional supplements (synbiotics), carrier abilities for bioactive compounds, drug release, encapsulation of pathogens or nucleic acids, and for pollutant removal from water and wastewaters (Table 2.16). There is also a constant need for the development of efficient vaccines and delivery systems to prevent and control the emerging and re-emerging infectious diseases in aquaculture. There are innumerable infectious diseases for which the development of efficient vaccines has been difficult to achieve. The failure is mainly due to the inability to design vaccines evoking appropriate immune responses. The use of chitosan-based nanoparticles has provided a tremendous opportunity to design vaccine delivery systems that are efficient in targeted delivery, providing stability to antigens, and act as efficient adjuvants. Many of the

Table 2.16 Applications of chitosan in aquaculture

Topic	Form	Applications
Quality water Nutrition Functional foods Nutritional supplements Probiotics/Prebiotics Controlled release of compounds Treatment of seafood effluents	Powder Microsphere Bead	Removal of organic compounds and inorganic nutrients; removal of bacteria, of ammonia Functional food; to enhance gelling properties Micro-carrier abilities for bioactive compounds: proteins, pigments Probiotics to improve feed conversion, growth rates, weight gain, immune system and disease resistance of fish Microencapsulation of drugs and drug delivery; oral delivery (vaccination) Immuno-stimulant against bacterial diseases Antimicrobial, antioxidant and antioxidative stress
References		
Chung et al. (2005), Chung (2006), Borgogna et al. (2011), Cerezuela et al. (2011), Alishahi and Aïder (2012), Harikrishnan et al. (2012), Niu et al. (2013), Zaki et al. (2015), Lian et al. (2016), and Bernardi et al. (2018)		

nanoparticles are able to enter the antigen presenting cells by different pathways and induce appropriate immune responses to the antigen. Vinay et al. (2018) reviewed the use of chitosan for the delivery of fish vaccines and compared the potential of these delivery systems for the development of new vaccines against different fish pathogens.

2.3.10 Textile Industry

Applications of chitosan and its derivatives in textile industry are described in Table 2.17. Among the possible approaches initiated by the textile industry, the use of chitosan presents an innovative possible avenue for large scale development of bioactive textiles (Giri Dev et al. 2005; Enescu 2008; Sahan and Demir 2014; Gutiérrez 2017; Roy et al. 2017). Indeed, more research and practical use results indicate that chitosan might act as active compounds in textiles, e.g. as antimicrobial finishing of textiles, and cosmetotextiles. The characteristics of chitosan used in textile industry include cost-effectiveness, non-toxic, biocompatible, biodegradable, antimicrobial activity, antistatic activity, chelating property, deodorizing property, film-forming ability, chemical reactivity, dyeing improvement ability, thickening property, and also wound healing activity. Although the antimicrobial activity of chitosan is well document in the literature, its mode of action is yet not fully understood (Islam et al. 2013). There are many possibilities for the development of new textile and cosmetic products containing chitosan-based nanoparticles with advanced properties (UV-blocking, water repellence, self-cleaning). Their applications seem promising.

Table 2.17 Applications of chitosan and its derivatives in textile industry

Topic	Forms	Applications
Textiles Functional textiles Cosmeto-textiles Medical textiles	Microcapsule Gel Gelatinous dispersion Coating Fiber	Dye-binder for textiles Impregnated textile materials Binding agent for non-woven Surface modification of textiles Textiles with anti-bacterial properties Textile printing and antimicrobial finishing Textile preservative and deodorant agent Non-allergenic fibers Sanitary fibrous products Surgical threads
References		
Giri Dev et al. (2005), Enescu (2008), Ummu Habeeba et al. (2007), Crini et al. (2009), Francesko et al. (2010), Islam et al. (2013), Şahan and Demir (2014), Hamed et al. (2016), Voncina et al. (2016), Gutiérrez (2017), and Roy et al. (2017)		

Table 2.18 Applications of chitosan and its derivatives in pulp and paper industry

Topic	Form	Applications
Papermaking industry Pulp and paper Water treatment Papermaking-related industries	Coating Powder Nanoparticle	Wet strength agent; strengthening additive Retention and drainage agents Paper sizing and finishing Surface coating application: coated papers with antibacterial and antimicrobial properties Confer strength to paper against moisture Biodegradable packaging for food wrapping Wrapping and toilet paper Chromatography paper Card board Carbonless copy paper Modification of cellulose fibers Photochromic paper Papermaking wastewater treatment
References		
Muzzarelli (1983), Struszczyk (2002), Crini et al. (2009), Cheba (2011), Samyn et al. (2018), and Song et al. (2018)		

2.3.11 Pulp and Paper Industry

Applications of chitosan in pulp and paper industry are described in Table 2.18. The first use of chitosan in the papermaking industry was reported in 1936 (Struszczyk 2002). The main use was to improve wet-strength of paper. Chitosan as functional material is also able to interact with cellulose pulp during the formation of paper and to be film-forming to offer cohesive resistance to rupture (Song et al. 2018). This biopolymer is also non-toxic, biodegradable and eco-friendly in order to facilitate compliance with environmental regulations. Chitosan as chelating and complexing agent is also used in the decontamination of pulp and paper wastewaters for removal of lignin, color and undesired contaminants, and for the decrease in total organic carbon and chemical oxygen demand. Samyn et al. (2018) recently reviewed the use of nanoparticles and nanostructured materials in papermaking.

2.3.12 Biotechnology

Some applications of chitosan in biotechnology are described in Table 2.19. Numerous enzymes (lysozyme, urease, *Escherichia coli* cells, amylases) were immobilized with chitosan. They are entrapped and absorbed in the macromolecule chains. In biochemistry, chitosan is used as a support for enzymes, mainly by cross-linking reactions. Chitosan and its derivatives have also shown biotechnology applications as biosensors and biodevices. Depolymerization and de-N-acetylation of chitin by chitinases and deacetylases generates a series of derivatives such as

Table 2.19 Applications of chitosan and its derivatives in biotechnology

Topic	Form	Applications
Enzymology Enzyme technology Separation Bioseparation Biomembranes Electrodes Biosensors	Powder Bead Microsphere Nanoparticle Sponge Membrane	Enzyme and cell immobilization Cell recovery Cell-stimulating materials Protein separation Matrix for affinity and gel permeation chromatography Electronic devices, biosensor construction Metabolic analysis of biological fluids
References		
Zikakis (1984), Struszczyk (2002), Krajewska (2005), Wang (2012), Suginta et al. (2013), Philibert et al. (2017), and Grifoll-Romero et al. (2018)		

chitooligosaccharides which find numerous applications in biotechnology as recently reviewed by Grifoll-Romero et al. (2018).

2.3.13 Chemistry

Table 2.20 describes some applications of chitosan in chemistry. Chitosan is the object of numerous studies concerning applications in chromatography, green chemistry, catalysis, membrane technology and electrochemistry. It has been proposed in thin layer chromatography for separation of nucleic acids and in green chemistry for the generation of green solvents. It is believed that chitosan will play a very important role in these new developments. The utilization of chitosan as a catalyst support is also of particular interest. This application combines several up-to-date techniques (freeze drying, utilization of supercritical CO_2) to increase the surface exchange capabilities and/or utilization of ionic liquids. Chitosan contributes to the implementation of green chemistry principles. The state of the art review of the design of electrochemical biosensor applications based on chitosan and chitin was presented by Suginta et al. (2013) and comprehensively discussed. Membranes are produced by casting chitosan solutions either alone or with suitable ingredients (reagents, polymers) to give desired properties for target applications. Preparation and application of chitosan-based adsorptive membranes for separation and for water purification were reviewed by Salehi et al. (2016) and by Thakur and Voicu (2016), respectively. Chitosan has been proposed for the separation of organic liquid mixtures using evapomeation membranes. This membrane separation technique makes use of the advantage of pervaporation and simultaneously removes a fault of pervaporation. The main applications of chitosan membranes are their use in biomedicine and biotechnology. Albumin blended chitosan membranes have been used in hemodialysis, artificial skin and also drug targeting. Further developments are expected in the near future in these domains (Galiano et al. 2018).

Table 2.20 Applications of chitosan and its derivatives in chemistry

Topic	Form	Applications
Analytical chemistry	Solution	Analytical reagent
Chromatography	Ionic liquids	Reverse osmosis
Dialysis	Powder	Permeability control
Reverse osmosis	Film	Solvent separation
Ultrafiltration	Coating	Organic/organic separation
Gas permeation	Fiber	Alcohol/water selective pervaporation
Pervaporation	Membrane	Transport direction of target molecules
Evapomeation technique	Composite	Membranes for lithium batteries
Carrier transport membranes	Blend	Biosensors, electrochemical devices
Green chemistry	Sensors	CO_2 recovery
Green solvents		Alternative solvents in catalytic organic reactions
Catalysis/Biocatalysis		
Adhesives		Adhesive role between metallic surfaces
Corrosion protection		
Polymer science		Corrosion protection of aluminum
Click chemistry		Ionic liquids
Electrochemistry		Deep eutectic solvents
References		
Goosen (1997), Guibal (2005), Krajewska (2005), Macquarie and Hardy (2005), Li et al. (2008), Crini et al. (2009), Cheba (2011), Dash et al. (2011), Suginta et al. (2013), Mati-Baouche et al. (2014), Carneiro et al. (2015), Salehi et al. (2016), Thakur and Voicu (2016), Osman and Arof (2017), Argüelles-Monal et al. (2018), Galiano et al. (2018), Marpu and Benton (2018), Xie and Yuan (2018), and Zdanowicz et al. (2018)		

2.3.14 Environmental Chemistry

A growing number of papers have been published since the 1980s concerning chitosan for applications in environmental chemistry (Table 2.21). This natural polymer possesses several intrinsic characteristics that make it an effective material for environmental purposes. Its use is justified by four important advantages:(i) its relatively low cost compared with commercial activated carbon or organic resins, (ii) its outstanding pollutant-binding capacities and excellent selectivity, (iii) its versatility, and (iv) its possible biodegradability after use. Indeed, one of the major applications of chitosan is based on its great ability to tightly bind a whole range of pollutants. In addition, chitosan and its derivatives can be used in soluble or insoluble forms including gels, beads, sponges, films and membranes, and fibers through coagulation-, filtration-, flocculation- or adsorption-oriented processes. In a previous review, Crini and Badot (2008) comprehensively discussed the development of chitosan-based materials (grafted and crosslinked derivatives) used as useful adsorbent polymeric matrices for dye removal. Their review highlighted results that have been obtained in their decolorizing application as biosorbents. The effects of various parameters such as chitosan's characteristics, the activation conditions, the process variables, the chemistry of the dye and the experimental conditions used in batch systems, on biosorption were presented and discussed. The authors also reviewed the various adsorption mechanisms involved. They concluded that the biosorbents

Table 2.21 Main applications of chitosan and its derivatives in environmental chemistry (selected reviews)

Topic	Form	Applications
Water treatment Wastewater treatment Coagulation Flocculation Adsorption Biosorption Solid-phase extraction Filtration *Membrane filtration* Remediation Biological denitrification	Solution Gel/hydrogel Powder Microsphere Bead Nanoparticle Fiber Hollow fiber Membrane Sponge	Coagulant/Flocculant: clarification of drinking water, pools and spas, wastewaters Adsorbent/Biosorbent: recovery of precious metals; metal chelation; dye removal; removal of pesticides, phenol derivatives, PCB and radioisotopes Interactions with proteins and amino acids Reduce odors Antifouling agent Polymer-assisted ultrafiltration Sludge treatment and dehydration agent Drilling muds
References		
Hirano (1997), Varma et al. (2004), Crini (2005, 2006, 2015), Krajewska (2005), Tang et al. (2007), Gérente et al. (2007), Alves and Mano (2008), Crini and Badot (2008), Li et al. (2008), Bhatnagar and Sillanpää (2009), Elwakeel (2010), Sudha (2011), Rhazi et al. (2012), Ravichandran and Rajesh (2013), Vakili et al. (2014), Liu and Bai (2014), Boamah et al. (2015), Yong et al. (2015), Azarova et al. (2016), Barbusinski et al. (2016), Kos (2016), Ahmad et al. (2017a, b), Crini et al. (2017), Sudha et al. (2017), Nechita (2017), Alaba et al. (2018), de Andrade et al. (2018), Desbrières and Guibal (2018), El Halah et al. (2018), Pakdel and Peighambardoust (2018), Van Tran et al. (2018), and Wei et al. (2018)		

were efficient in pollutant removal with the additional advantage of being cheap, non-toxic and biocompatible. More recently, Kyzas et al. (2017), Desbrières and Guibal (2018) and Pakdel and Peighambardoust (2018) also indicated that biosorption onto chitosan is a promising alternative to replace conventional adsorbents used for decolorization purposes, and metal and organic removal. Chitosan-based versatile materials are also widely proposed in clarification and water purification, and water and wastewater treatment as coagulating and flocculating agents (Crini et al. 2009). As ecofriendly materials, they can be a potential substitute for metallic salts and synthetic polyelectrolytes in the treatment of water for the removal of both particulate and dissolved substances. However, despite a large number of studies on the use of chitosan for pollutant recovery in the literature, processes are basically at the stage of laboratory-scale study in spite of unquestionable progress. Indeed, these research fields for chitosan have failed to find practical applications on the industrial scale. The actual applications in industry remain rather rare, e.g. Pennofloc™ for water clarification, ChitoVan™ for biofiltration, as concurrent flocculating and adsorbing agents are cheaper. Even if chitosan shows better performances in term of pollutant elimination, the conventional products are sufficient to fulfill current regulatory frameworks.

2.3.15 Miscellaneous

Table 2.22 describes applications of chitosan in miscellaneous domains. Due to its optical characteristics, film-forming ability and reactions with silver complexes, chitosan and derivatives found applications in photography. In color photography, chitosan has been proposed as a fixing agent for acid dyes in gelatin and also acts as an aid to improve diffusion (Dutta et al. 2004). Ecological concerns about antifouling paints containing non-green tin and copper compounds have highlighted the need for environmentally friendly alternatives (Pelletier et al. 2009; Banerjee et al. 2011). Novel chitosan-based technologies may prevent fouling by means of unfavorable surface chemical and physical properties or by concentrating antifouling compounds around surfaces. A prototype of antifouling paints was proposed as a possible replacement for traditional antifouling paints by Heuser and co-workers (Heuser et al. 2009; Heuser and Cárdenas 2014). In cement industry, chitosan has been proposed as water proofing and water repellent (Cheba, 2011). Chitosan has been also explored in the production of plastics (Galiano et al. 2018) or in the stabilization of photonic materials (Marpu and Benton 2018).

Table 2.22 Applications of chitosan in miscellaneous domains

Field	Main form	Applications
Photography	Solution	Photographic paper
Detergents	Powder	Film forming ability
Surfactants	Coating	Fixing agent for color photography
Paints	Nanoparticles	Color film
Adhesives	Nanoclusters	Nanoimprinting lithography
Wood industry		Antifouling paints
Cement industry		Thermosensitive materials
Leather		Improvement of wood quality
Plastics		Wood adhesive
Bioplastics		Protection of wood (fungicide)
Cigarette industry		Lithium batteries
Semiconductors		Specific targeting
Luminescent nanoparticles		Cellular imaging
Photonics		Bio-imaging and cancer research
Imaging applications		Bioconjugation to biomolecules
Quantum dots		Sensors and biosensing
Carbon dots		Temperature sensing
		Detection
References		
Struszczyk (2002), Crini et al. (2009), Elsabee et al. (2009), Heuser et al. (2009), Pelletier et al. (2009), Desbrières et al. (2010), Banerjee et al. (2011), Cheba (2011), Heuser and Cárdenas (2014), Mati-Baouche et al. (2014), Al-Naamani et al. (2017), Argüelles-Monal et al. (2017), Galiano et al. (2018), and Marpu and Benton (2018)		

2.4 Conclusion

Isolated from mushrooms in 1811 by Braconnot, chitin is the first polysaccharide identified by man, preceding cellulose by about 30 years, while its deacetylated derivative was discovered by Rouget in 1859. In 1894, Hoppe-Seyler confirmed that the product was deacetylated chitin and named it chitosan. Since, these biopolymers, in particular chitosan, have considerably attracted the interest of scientists and industries in different disciplines including health science, agriculture, chemistry, biochemistry, and environment. The main reasons for this growing interest are its intrinsic biological properties and its versatility as biomaterial. Today, chitosan continue to offer new horizons to scientists and industrials with a wide range of possible modifications and forms. In this chapter, we aim to present an overview of the state of the art in the applications of chitosan, based on a substantial number of relevant references published in the last two decades on various biological, biotechnological, chemical, and physical topics for both academic and industrial applications. Of course, this is an ambitious project and the examples discussed in this chapter are not exhaustive but clearly demonstrate the benefit of chitosan in many fields. The main markets for chitosan are food, pharmaceutical and cosmetic industries. The chitosan market is expected to grow rapidly because of increasing consumption not only in cosmetics but also in water treatment, beverage industry, and nutrition. Indeed, nutraceuticals and cosmeceuticals are growing actual markets. There is also an increased demand for chitosan in emerging countries. Therapeutic and biomedical products are also expected to have a positive impact on the market. It is important to point out that, although numerous papers and patents have been reported in the last two decades, the applications of chitosan in the biomedical area are still limited, mainly due to the extreme difficulty to access sufficient purity and source reliability of the biopolymer. In addition, the development of new materials are rather limited, mainly due to their cost, which remains higher than that of petroleum based polymers with similar properties. Finally, *in vivo* studies are currently limited. Further industrial developments are expected in the near future in the following domains: anticancer drugs, gene delivery, catalysis, sensor applications, wrapping materials and packaging, cosmetotextiles, and bio-imaging.

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Chapter 3
Biocatalytic Production of Hetero-Chitosan Oligosaccharides as Anti-oxidants

Swati Jaiswal, Pushplata Tripathi, and Sujata Sinha

Abstract Abundantly available chitin/chitosan and their derivatives are full of useful bioactivities. They have numerous applications in industries like food, wastewater treatment, pharmaceuticals, agriculture, cosmetics etc. However, their insolubility in water plays spoilsport in way of their use as cost-effective biomolecules for various sectors. Breakage of chitosan to smaller oligosaccharides solves this problem to larger extent preferably using highly specific enzymes. It is well known that that bioactivities of oligosaccharides improve upon hydrolysis to lower molecular weight chitosan i.e. chitooligosaccharides. Availability and production of anti-oxidant chitooligosaccharides by non-chemical approach is desirable for consumer satisfaction. Bioprocessing of chitin/chitosan generated from marine waste to be used as bioactive chitooligosaccharides, can reduce both environmental and human health hazards to a great extent.

Here we review (1) biocatalytic approaches for chitooligosaccharides production, (2) bioprocess strategies for large scale production, (3) functionalization and (4) anti-oxidant activity of chitooligosaccharides. Specific and non-specific biocatalysts are used for chitooligomer preparation either by hydrolysis and transglycosylation approaches. Cellulase enzymes have been found to be most frequently used non-specific enzymes for chitosan hydrolysis but microbial chitosanases show excellent performance for chitooligosaccharides production both in terms of yield and specificity. Transglycosylation also have been found to be promising for chitooligosaccharides production especially at small scale. Combination reactors have been found to be most suitable for upscaling of chitooligomer production. Immobilized packed column with ultrafiltration membrane reactors are used for simultaneous hydrolysis and separation of chitooligomers. Chemically synthesized derivatives of chitooligomers have been reported in many studies by introducing carboxyl, quaternized amino, amino ethyl, sulfate, gallyl and many more groups. Amino ethyl, Gallyl, sulphated,

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phenolic acid conjugated and carboxylated derivatized chitooligomers have shown anti-oxidant activity. Anti-oxidant activity of chitooligomers and relation with their structure and polymerisation has been well established. Chitooligomers longer than trimer show good activity while best activity has been reported in degree of polymerisation from 10 to 12. Acetylation of chitooligomers leads to improvement in anti-oxidant activity than their deacetylated version.

Keywords Anti-oxidant · Oligosaccharides · Chito-oligosaccharides · Chitosanase · Chitin · Chitosan · Radical-scavenging · DPPH · Bioactivity

Abbreviations

ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CAT	chloramphenicol acetyltransferase
COS	chitooligosaccharides
DCFH-DA	dichloro-dihydro-fluorescein-diacetate
DD	degree of deacetylation
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DNA	deoxyribonucleic acid
DNA	deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ESR	electron spin resonance
FRAP	ferric reducing power
GSH-PX	glutathione peroxidase
KDa	kilo Dalton
PANNFM	polyacrylonitrile nanofibrous membrane
QCMCOS	quaternised carboxymethyl chitooligosaccharide
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TBHQ	tertiary butyl hydroquinone

3.1 Introduction

Chitin is the second most abundant renewable biopolymer after cellulose in nature which could be used as starting material for various industries (Kaur and Dhillon 2015). Chitin and chitosan have been converted to oligosaccharides because lower solubility of chitosan in water possess difficulty in their application for various purposes and oligosaccharides are shown to have better bioactivity than their chitosan/chitin polymers. Chitosans with degree of polymerization less than 20 and an average molecular weight less than 3.9 KDa are called chitosan oligosaccharides (Mourya

et al. 2011). Chitooligosaccharides can be homochitooligomers or heterochitooligomers. Homochitooligomers contain either only glucosamine or D-glucosamine units, whereas heterochitooligomers contain both types of monomers and can be a mixture of various oligomers having various degree and position of polymerisation/acetylation. Hetero-chitooligosaccharides with degree of polymerisation less than 10 are soluble in water but for chitooligosaccharides having degree of polymerisation more than 10, solubility depends on pH of the solution and degree of acetylation. Pharmaceutical companies, food industries and researchers preferably uses chitooligosaccharide in hetero form (Il'ina and Varlamov 2015). Chitooligosaccharides have same or variation in degree of acetylation and the sequence of acetylated/deacetylated residues, however these can be considered as homologs (Kim 2011).

Chitin/chitosan conversion to their oligosaccharides can be achieved physically, chemically or bio-catalytically (Aam et al. 2010). Ultrasonic and gamma irradiation have been used for physical depolymerisation. Fully deacetylated chitosan was depolymerised with ultrasonic irradiation to produce chitosan oligos with degree of polymerisation between 2 and 11 and maximum concentration of trimers (Popa-Nita et al. 2009). Gamma irradiation has been used for reducing the viscosity of chitosan in acetic acid solution and producing dimers, trimers and tetramers of chitin/chitosan polymer (Choi et al. 2002). Chitosan hydrolysis using various acids like hydrochloric acid, electrolyte acid, nitrous acid, phosphoric acid, fluoric acid etc. and hydrogen peroxide or persulfate using oxidative/reductive methods have also been demonstrated (Lodhi et al. 2014). Milder acids like lactic acid, trichloroacetic acid, formic acid, acetic acid etc. has also been studied for their degradative action on chitosan (Ando and Kataoka 1980; Yamaguchi et al. 1982; Il'ina and Varlamov 2004). However, chemical reactions are difficult to control leading to synthesis of spurious or secondary products which hinder the downstream processing and therefore are not eco-friendly. Enzymatic processes are considered to be most feasible and attractive for chitooligosaccharide preparation either by hydrolysis of chitin/chitosan and related substrates or by synthesis of larger oligomers by transglycosylation methods. Biocatalytic methods reduce the use of toxic chemicals, are easier to control and do not generate any harmful waste as in case of chemical methods. Various specific and non-specific biocatalysts used for chitooligosaccharide production and their reported anti-oxidant activity has been covered in this chapter (Fig. 3.1).

3.2 Biocatalysts of Chitosan Oligosaccharides

3.2.1 Specific Biocatalysts

Chitosan enjoys broad substrate specificity as it is susceptible to number of carbohydrases, proteases and chitinases reported from fungi and bacteria (Kim and Rajapakse 2005; Sinha et al. 2016a). Microbial chitosanases from fungi and bacteria have shown excellent performances in hydrolysing chitosan of various degree of deacetylation and these enzymes have also been reported in virus, animals and plants (Hamed et al. 2016). However, chitosanases reported from microbial sources

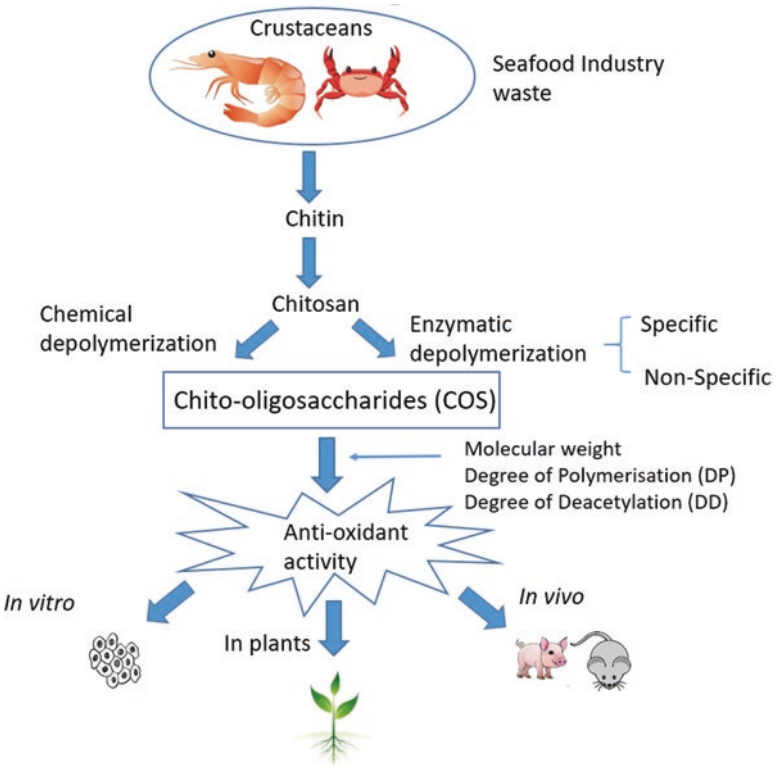

Fig. 3.1 Biocatalytic production of chitooligosaccharides and their anti-oxidant activity. Chitin from waste from seafood industry is the source of chitosan which is depolymerized (enzymatic or chemical) to produce chitooligosaccharides with a range of molecular weight, degree of polymerization and deacetylation. These chitooligosaccharides have been found to exert antioxidant activity in vitro, in vivo and in plants

are very few and are expensive to be used at industrial scale due to high cost of extraction, concentration and purification. There are specific and non-specific enzymes which affect the types and structure of glycosidic bonds in chitosan hydrolysis. Random distribution of four types of glycosidic bonds in the structure of chitosan determines their hydrolysis by enzymes. These could be between two deacetylated units (D-D), one acetylated and one deacetylated (A-D), one deacetylated and one acetylated (D-A) and two acetylated units (A-A) and their hydrolysis depends on presence of reducing/non-reducing ends and degree of deacetylation. Lysozyme from egg white has been found to be specific towards two acetylated (A-A) units and *Bacillus* chitosanase has been found to be specific towards glycosidic bonds two deacetylated units (D-D) (Vårum et al. 1996). Chitinases act on partially deacetylated chitosan by detecting the presence of N-acetyl glucosamine (GlcNAc) moiety in the sequence of chitosan (Aiba 1994). Chitosan and other non-specific enzymatic hydrolysis with respect to microbial source of enzyme and product obtained, have been summarised in Table 3.1.

Table 3.1 chitooligosaccharide production as a result of chitosan specific and non-specific enzymatic hydrolysis, their sources and reported anti-oxidant activity

Enzyme	Microbial source	Chitooligosaccharides product	Anti-oxidant activity	References
Specific enzyme				
Chitosanase	Streptomyces sp	Dimer to hexamer	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity	Sinha et al. (2012a) and Sinha et al. (2014)
Chitosanolytic enzymes	*Metarhizium anisopliae*	Dimer to hexamer	Not studied	de Assis et al. (2010)
Chitosanase	*Purpureocillium lilacinum* CFRNT12	Dimer to hexamer	Not studied	Nidheesh et al. (2015)
Chitosanase	Bacillus sp	10 KDa, 5 KDa, 3 KDa, 1 KDa	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, Alkyl radical assay, superoxide radical, hydroxyl radical	Park et al. (2003)
Chitosanase	Commercial	Details not given	Improved total anti-oxidant capacity and activity of SOD (superoxide dismutase)	Yuan (2009)
Chitinolytic enzyme	Chitiniphilus sp. LZ32	Dimer, trimer, tetramer, pentamer, hexamer	Reducing power of chitooligosaccharide, superoxide, hydroxyl radical scavenging	Zhang et al. (2017)
Non-specific enzyme				
Glycosyl transferase (Branchzyme) Free and immobilized form	Not known	2-20 polymerisation with high conc of 3-8 mers	Not reported	Montilla et al. (2013)
Lysozyme, papain, cellulase	Papain showed highest mol wt reduction	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, metal chelating activity	(0.003% w/w) compared between three enzyme	Laokuldilok et al. (2017)
Cellulase	Commercial	Hydrogen peroxide, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, ferrous ion chelation	Negative correlation between mol wt of COS and anti-oxidant activity was established	Chang et al. (2018)

3.2.2 Non-Specific Enzymatic Hydrolysis

As specific enzymes are not available in bulk for commercial preparation and are not cost effective hence non-specific biocatalytic preparations of chitooligomers are being explored for this purpose. Lipases, cellulases (Wu and Tsai 2004), papain, lysozyme, hemicellulases, protease, pectinases, pepsin, pronase, chitinases and many other non-specific enzymes have been reported for hydrolysis of chitosan (Abdel-Aziz et al. 2014). Cellulase is the frequently used non-specific enzyme being reported for chitosan hydrolysis and this is explained by similarity between structure of chitin, chitosan and cellulose due to presence of β, 1-4 glycosidic bond between glucose subunits. The presence of acetamide group in chitin and amino group in chitosan at C-2 hydroxyl position is found to have no role in enzyme substrate reaction. Wide variety of cellulases, especially bifunctional chitosanase-cellulase from various microbial sources have been reportedly used for production of chitooligomers from chitosan (Xia et al. 2008). All these enzymes belong to glycosyl hydrolase family mainly GH-5, GH-7 and GH-8 and few have been found to be superior to even chitosanases enzymes for chitosan hydrolysis.

3.2.3 Transglycosylation Activity for Synthesis of Chitooligosaccharides

Apart from hydrolysis of chitosan, chitooligomers are also synthesised using transglycosylation activity of biocatalysts. Chemical and enzymatic methods of chitooligosaccharides synthesis have been proposed and has been reviewed extensively (Yang and Biao 2014; Li et al. 2016). Large number of steps related to protection and deprotection make chemical glycosylation methods cumbersome as steps increases with the size of oligosaccharides, so synthesis of trimer and larger oligosaccharides are not considered feasible. However, biocatalytic methods allow regioselectivity, milder reaction and hassle-free ways (no protection and deprotection step) of glycosidic bond formation. Formation of new glycosidic bonds between donor and acceptor saccharides can also be established by few glycosyl hydrolases apart from their usual activity of glycosidic bond hydrolysis (Li et al. 2016). Active-site architecture of transglycolytic enzymes in their full efficiency hinders correct positioning of water molecule and promotes/favours binding of incoming carbohydrate molecule through strong interaction of aglycon subsites. Released chitooligosaccharides is transferred to suitable acceptor to form a new glycosidic bond to synthesize chitooligosaccharide. Stereo- and size specific preparation of higher chitooligosaccharide have been achieved using this activity of chitosanase/chitinolytic enzymes and other glycosidases. An exo-chitosanase enzyme having transglycosylation activity was isolated from *Aspergillus fumigatus* IIT-004 which was immobilized on nanofibers and employed for chitosan hydrolysis. However, synthesis of chitodimer was also achieved using this enzyme when reaction conditions were

changed (Sinha et al. 2016b). Purified chitinase from *Trichoderma reesei* KDR-11 has been shown to convert dimer and hexamer of N-acetyl glucosamine $(GlcNAc)_2$ (55.7%) and $(GlcNAc)_6$ (39.6%) from tetramer of N-acetyl glucosamine $(GlcNAc)_4$ (100%) using a transglycosylation reaction (Usui et al. 1990). Lysozyme from hen egg-white lysozyme has been used for synthesis of chitooligosaccharide (4-12) polymerisation (Akiyama et al. 1995).

3.2.4 Bioprocess Strategies for Chitooligosaccharides Production/Synthesis

Upscaling of COS production has been tried mainly in three types of bioreactor settings like batch, column and ultrafiltration reactor (Vidanarachchi et al. 2010). Batch reactor is most common where enzyme (chitosanase from *Bacillus pumilus* BN-262) is mixed with substrate (1% chitosan) and glycosidic bonds are allowed to break under optimized pH, temperature and time. Nonetheless, it has certain drawbacks like lower yield, lack of continuous production and higher cost due to lack of enzyme reusability. Packed immobilized enzyme in a column reactor through which substrate is passed continuously, has been suggested for continuous production but its use has been limited by poor affinity of immobilized enzyme towards substrates. Enzyme immobilization has been tested with various carriers for column packing but chitosanase bonded on chitin has shown better activity than other matrices (Kim and Rajapakse 2005). Ultrafiltration membrane with cut off of 3 KDa has been used for production of relatively higher oligomers of chitosan (trimer to hexamer) (Jeon and Kim 2000). Eleven batches of hydrolysis could be achieved with same amount of enzyme used in batch condition, thus at relatively lower cost, chitooligosaccharide with higher degree of polymerisation (chitotrimer to chitohexamer) could be achieved. In continuous reactor, a dual reactor system was proposed where ultrafiltration membrane was attached to chitin packed column with immobilized chitosanase enzyme system (Jeon and Kim 2000). Chitosanase enzyme was physically adsorbed on the chitin but showed less affinity and lower reaction rate towards substrates than free enzyme. Optimized permeation rate of 4 ml/min was determined where the 80% of product contained larger chitooligosaccharides (trimer to hexamer). Membrane of 10 KDa was used in ultrafiltration membrane reactor for selective fractionation of chitooligosaccharide which resulted from hydrolysis of partially hydrolysed chitosan and this was controlled by changing flow rate. Monomer production from chitooligosaccharide was stopped by controlling the chitosan hydrolysis which in turn halted product inhibition. In this study, membrane fouling in ultrafiltration membrane was removed by partially hydrolysing chitosan before applying to the reactors. Chitooligosaccharides preparation by continuous hydrolysis of chitosan in ultrafiltration membrane reactor along with immobilized column reactor utilising *Bacillus* chitosanase has also been reported from a different study where the product was used for radical scavenging activity studies (Park et al. 2003).

A chitosanolytic α-amylase enzyme from *Bacillus amylolyquefaciens* was covalently immobilized on glyoxal agarose beads and was assessed in batch and fixed bed reactors for continuous production of chitooligosaccharide with activity recovery of 25% (Moriano et al. 2016). Here, improved thermostability of the immobilized enzyme and conversion yield of 73% was obtained. Also, chitotriose and chitobiose were found to be the major products and conversion yield dropped by an increase in the dilution rate. In yet another study, polyacrylonitrile nanofibrous membrane (PANNFM) based chitosanase enzyme from *Aspergillus* sp. was used for selective fractionation of chitodimer to hexamer by varying the reaction temperature (Sinha et al. 2012b).

3.2.5 Production of Functionalized Chitooligosaccharides

Not only chitooligosaccharide but their derivatives have also shown antioxidant activity in different biological systems (Table 3.2). Various functional groups like hydroxyl and amino groups in the chitosan backbone have been added by enzymatic approaches to improve its application in various fields as it leads to changes in physicochemical properties. Amide coupling reaction has been used for conjugates preparation like phenolic and gallic acid conjugates (Liaqat and Rengin 2018). Phenolic acid compounds have tendency to donate H atom which enhanced the potential of conjugated anti-oxidant compounds, similarly, gallic acid conjugated chitooligosaccharides have also shown anti-oxidant capacity (Vo et al. 2017) Also, quaternization, alkyltion, thiolation, hydroxyalkylation, carboxyalkylation are some of the methods by which chitooligosaccharides can be modified (Mourya and Inamdar 2009). In a study on functionalized chitooligosaccharide, it was observed that chitooligosaccharides have better anti-oxidant activity than their O- and N-carboxymethyl substituted counterparts, while reducing power was greatest in O-carboxymethyl substituted chitooligosaccharide. In case of quaternized carboxymethyl chitooligosaccharide (QCMCOS), anti-oxidant activity was directly related to degree of substitution. Substitution with quaternary ammonium and carboxymethyl group also enhanced their thermal stability and degree of crystallinity (Li et al. 2012).

3.3 Anti-Oxidant Activity

Antioxidant molecules play an important part in neutralization of oxidative stress in living systems since they bind free radicals. Free radicals, also known as Reactive Oxygen Species or ROS, are chemical species with an unpaired electron in its outer orbital and therefore they are highly reactive e.g. hydroxyl free radical (OH°). ROS harms the cell membrane by lipid peroxidation and impairs cellular machinery by DNA and protein oxidation. Free radicals are produced under normal as well as pathological conditions and levels of free radicals are balanced by the endogenous

Table 3.2 Preparation of COS conjugates by functionalization using various derivatives and their anti-oxidant activity

Derivative	Preparation Method	Biological System	Antioxidant capacity assay	References
Carboxyl	Chemical synthesis	Cell free system	Ferrous ion chelating activity 1,1-diphenyl-2-picrylhydrazyl (no) radical scavenging Carbon-centered radicals scavenging by electron-spin resonance (ESR) Hydroxyl radical scavenging	Huang et al. (2006)
Amino	Chemical synthesis	Cell free system	Ferrous ion chelating activity 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging Carbon-centered radicals scavenging by electron-spin resonance (ESR) Hydroxyl radical scavenging	Huang et al. (2006)
Gallic acid	Chemical synthesis	Human chondrosarcoma (SW1353 cells)	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Superoxide anion radical scavenging activity Hydroxyl radical scavenging activity Reactive oxygen species (ROS) detection by Dichloro-dihydro-fluorescein-diacetate Dichloro-dihydro-fluorescein-diacetate (DCFH-DA)assay	Ngo et al. (2011)
N-Maleoyl chitosan and N-succinyl	Chemical synthesis	Cell free system	Superoxide anion radical scavenging activity Hydroxyl radical scavenging activity and ferric reducing power (FRAP)	Sun et al. (2011)
Phenolic acid conjugate	Chemical synthesis	Cell free system	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Hydroxyl radical scavenging activity Nitric oxide radical scavenging assay	Eom et al. (2012)
QCMCOS (quaternized)	Chemical synthesis	Cell-free system	Hydroxyl radical scavenging/ Fe^{2+} chelation assay, reducing power, superoxide anion free radical assay	Li et al. (2012)
Aminoethyl	Chemical synthesis	RAW 264.7 cells	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Intracellular glutathione level	Ngo et al. (2012)

(continued)

Table 3.2 (continued)

Derivative	Preparation Method	Biological System	Antioxidant capacity assay	References
Aminoethyl	Chemical synthesis	BV-2 cells	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Lipid peroxidation assay Protein oxidation assay	Ngo et al. (2012)
Sulphated	Chemical synthesis	MIN6 cells	Reactive oxygen species detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Measurement of enzymes such as superoxide dismutase, chloramphenicol acetyltransferase (CAT), glutathione peroxidase (GSH-PX)	Lu et al. (2012)
Hydroxy benzaldehyde	Chemical synthesis	BV-2 cells	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) measurement of NF-κB and Nrf2 proteins deoxyribonucleic acid (DNA) oxidation assay	Oh et al. (2017)

Abbreviations: *DPPH* 2,2-Diphenyl-1-picrylhydrazyl, *QCMCOS* quaternised carboxymethyl chitooligosaccharide, *ESR* Electron spin resonance, *DCFH-DA* Dichloro-dihydro-fluorescein-diacetate, *FRAP* ferric reducing power, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *CAT* Chloramphenicol acetyltransferase, *GSH-PX* Glutathione peroxidase, *NF-κB* Nuclear Factor kappa beta, *Nrf2* Nuclear factor (erythroid-derived 2)-like 2

antioxidants produced by the living systems during normal physiological state (Valko et al. 2007). Imbalance between their level leads to damage to the biological components which is one of the underlying cause of diseases and disorders such as cancer, arteriosclerosis, stroke, heart attack, Alzheimer's, ageing etc. (Pham-Huy et al. 2008).

Initiation of free radical injury can be caused by ionising radiations, inflammatory conditions, excess metal ions in the body and drugs/chemicals such as acetaminophen, carbon-tetrachloride. Generally, these are overcome by body's defence mechanism which comprises of antioxidants (vitamins A, C, E, glutathione), enzymes (superoxide dismutase; catalase; glutathione peroxidase (GSH-PX) and metal carrier proteins (transferrin, ceruloplasmin) (Yu 1994). However, it has been postulated that external supply of antioxidants to the body will help in relieving it from diseases and disorders caused by oxidative stress (Sindhi et al. 2013). Thus, there is ongoing research towards discovery and synthesis of both artificial and natural antioxidant molecules which may assist patients in conquering their symptoms. Chitosan and chito-oligosaccharides have been extensively researched as natural antioxidants which are not only inexpensive but also biodegradable. The various antioxidant capacity assay along with their principle and end-product determination have been summarised in Table 3.3.

Table 3.3 Antioxidant assay, principle of the method and end product determination

Antioxidant capacity assay	Principle of the method	End-product determination
1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity	Antioxidant reaction with DPPH free radical which loses its violet colour	Colorimetry/ESR spectroscopy
ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) assay	Antioxidant reaction with the long-lived ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) radical	Colorimetry/ESR spectroscopy
Metal chelating activity	Chelation of ferrous ions by the inhibition of ferrozine–Fe^{2+} complex formation due to antioxidant	Colorimetry
FRAP (ferric reducing power)	Potassium ferricyanide reduction by antioxidants and subsequent reaction of potassium ferrocyanide with Fe^{3+}	Colorimetry
Superoxide anion radical scavenging activity	Superoxide anion production by a luminol-enhanced auto-oxidation of pyrogallol or by ultra violet (UV) irradiated riboflavin/ethylene diamine tetra acetic acid (EDTA) system followed by incubation with antioxidant.	Chemiluminescence quenching /ESR spectroscopy
Hydroxyl radical scavenging activity	Antioxidant capacity to quench OH radicals generated by a Co(II) based Fenton-like system	Loss of fluorescence of fluorescein/ESR spectroscopy
Carbon-centered radical spin adduct analysis	Carbon-centered radicals generated by AAPH (2,2-azobis(2-amidinopropane dihydrochloride)	ESR spectroscopy
TBARS (thiobarbituric acid reactive substances) assay	Hydroxyl radicals attack deoxyribose leading to the formation of thiobarbituric acid reactive substances (TBARS)	Colorimetry
Lipid peroxidation assay	Malondialdehyde (MDA) and other aldehydes are formed during lipid oxidation	Colorimetry
ROS (reactive oxygen species) detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) assay	Oxidation-sensitive dye Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) detects formation of intracellular ROS	Fluorimetry
Measurement of enzymes such as superoxide dismutase (SOD), chloramphenicol acetyltransferase (CAT), glutathione (GSH)	Enzymes involved in free radical defence mechanism of living cells	Enzyme activity measurement
Measurement of NF-κB and Nrf2 proteins	Role of NF-κB and Nrf2 proteins in redox balance maintenance	Western blot analysis

(continued)

Table 3.3 (continued)

Antioxidant capacity assay	Principle of the method	End-product determination
Measurement of mRNA expression of antioxidant enzymes (GPx1, GPx4, and Mn-SOD) or pro-inflammation cytokines, tumor necrosis factor (TNFα)	Mn and Cu/Zn-SOD (superoxide dismutase), chloramphenicol acetyltransferase (CAT), glutathione (GSH), glutathione peroxidase (GSH-PX) are antioxidant enzymes	Real time polymerase chain reaction (PCR)
DNA oxidation assay	Hydrogen peroxide mediated DNA oxidation is performed by reacting Fe(II) and H_2O_2 on genomic DNA which is inhibited by the antioxidant.	Agarose gel electrophoresis
Protein oxidation assay	Oxidation of cellular proteins by the Fenton reaction products produces carbonyl groups such as aldehydes and ketones in proteins	Spectroscopy

Abbreviations: *DPPH* 2,2-Diphenyl-1-picrylhydrazyl, *QCMCOS* quaternised carboxymethyl chitooligosaccharide, *ESR* Electron spin resonance, *DCFH-DA* Dichloro-dihydro-fluorescein-diacetate, *FRAP* ferric reducing power, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *CAT* Chloramphenicol acetyltransferase, *GSH-PX* Glutathione peroxidase, *NF-κB* Nuclear Factor kappa beta, *Nrf2* Nuclear factor (erythroid-derived 2)-like 2

3.3.1 Anti-Oxidant Activity of Chitooligosaccharides

In view of growing interest in identification of natural anti-oxidants, anti-oxidant activity of chitooligosaccharides has been explored in various biological systems (Table 3.4). Chitooligosaccharide and their conjugates possess greater anti-oxidant activity as compared to chitosan. Although, molecular mechanism of antioxidant activity of chitooligosaccharides is unclear but it is suggested that amino group in chitooligosaccharides react with unstable free radicals in order to make them stable, resulting in its anti-oxidant activity. Radical scavenging activity or anti-oxidant activity of chitooligosaccharide has been correlated with molecular weight, degree of polymerisation, degree/fraction of deacetylation and N-acetylation and chitosan substrate source (Anraku et al. 2018). In a systematic study on seven chitosan samples with molecular weight in the range of 2 to 300 KDa it was concluded that anti-oxidant properties of chitosan and chitooligosaccharide were inversely proportional to their molecular weight (Laokuldilok et al. 2017; Chang et al. 2018). Chitooligosaccharides with active hydroxyl and amino groups helps to scavenge free radicals and low molecular weight chitooligosaccharide with lower degree of polymerisation have more such groups available for reaction. Chitobiose and chitotriose were found to possess better anti-oxidant and reducing activity than chitooligosaccharides with higher degree of polymerisation (Chen et al. 2003). Chitooligosaccahrides with degree of polymerisation (10-12) show best anti-oxidant

Table 3.4 Molecular weight/degree of polymerisation and their *in vitro/in vivo* anti-oxidant activity of chitooligosaccharides

Biological System	Antioxidant capacity assay	MW, DD and DP of highest activity	References
In vitro			
Cell free system	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Hydroxyl radical scavenging activity Superoxide anion radical scavenging activity Carbon-centered radical spin adduct analysis	5–10 KDa 90% DD	Je et al. (2004)
Polymorphonuclear leukocytes	Superoxide radical Reactive oxygen species (ROS) detection by dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	1.1 KDa, degree of polymerisation DP = 7; DD 92.3%	Yang et al. (2006)
Cell free system	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Superoxide anion radical scavenging activity Hydroxyl radical scavenging activity Thiobarbituric acid reactive substances (TBARS) assay	Not defined	Rao et al. (2006)
Cell free system	Ferrous ion chelating activity 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Carbon-centered radical spin adduct analysis Hydroxyl radical scavenging activity	DD 76.54% Activity decreased with increased degree of substitution	Huang et al. (2006)
Rabbit neutrophils	Superoxide anion radical scavenging activity	DD 95%	Dou et al. (2007)
B16F1 cell line	Hydroxyl radical scavenging activity superoxide anion radical scavenging activity Carbon-centered radical spin adduct analysis Intracellular ROS detection by dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Intracellular glutathione (GSH) level	<1 KDa with DD 90%	Mendis et al. (2007)
Cell free system	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assay Superoxide anion radical scavenging activity Hydroxyl radical scavenging activity Metal chelating activity Scavenging of hydrogen peroxide	1.7 KDa with DD 47.9%	Feng et al. (2007)

(continued)

Table 3.4 (continued)

Biological System	Antioxidant capacity assay	MW, DD and DP of highest activity	References
Salmon tissue homogenate	1,1-diphenyl-2-picrylhydrazyl(DPPH)radical scavenging Thiobarbituric acid reactive substances (TBARS) assay	30 KDa, DD 84.71%	Kim and Thomas (2006)
ECV304 cell line	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	DP 2–6; DD 95%	Liu et al. (2009)
RAW 264.7 cell line	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Intracellular glutathione (GSH) level	DD <10%; MW 1–3 KDa	Ngo et al. (2009)
HepG2	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	Not defined	Cho et al. (2010)
Erythrocytes and bacteriophages	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assay hemolysis	<3 and <5 KDa; DD 80–85%	Fernandes et al. (2010)
HITT15 cell line	Reactive oxygen species (ROS) detection by dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	3–5 KDa	Karadeniz et al. (2010)
Cell free system	Hydroxyl radical scavenging activity superoxide anion radical scavenging activity Metal chelating activity	DD 85%	de Assis et al. (2012)
L02 cells	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Lipid peroxidation assay Intracellular glutathione (GSH) level	DD ≥95%; <1 KDa	Luo et al. (2014)
Cell free system and blood mononuclear cells	Hydroxyl radical scavenging activity superoxide anion radical scavenging activity 1,1-diphenyl-2-icrylhydrazyl (DPPH) radical scavenging activity	DP 2	Salgaonkar et al. (2015)
SH-SY5Y cells	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Superoxide anion radical scavenging activity Measurement of Nrf2 proteins	DP <10	Huang et al. (2015)
Cell free system	1,1-diphenyl-2-picrylhydrazyl((DPPH) radical scavenging activity, ABTS (2,2′-azino-bis-3-thylbenzthiazoline-6-sulfonic acid)assay FRAP (ferric reducing power)	<1 KDa	El-Sayed et al. (2017)
Cell free system	1,1-diphenyl-2-picrylhydrazyl(DPPH) radical scavenging activity Metal chelating activity FRAP (ferric reducing power)	5.1KDa; DD 90%	Laokuldilok et al. (2017)

(continued)

Table 3.4 (continued)

Biological System	Antioxidant capacity assay	MW, DD and DP of highest activity	References
In vivo			
Male albino rats	Lipid peroxidation assay	150 KDa; DD 82%	Koryagin et al. (2006)
Sprague-Dawley rats	Reactive oxygen species detection by dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	2.3 KDa; DD 92.7%	Yang et al. (2006)
Male Wistar rats	Measurement of enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) Lipid peroxidation assay	1.2 KDa; DD 90%	Yuan (2009)
Sows	Measurement of mRNA expression of several antioxidant enzymes GSH-PX (glutathione peroxidase), total-superoxide dismutase (SOD) and chloramphenicol acetyl transferaase (CAT) Lipid peroxidation assay	DP 2–7	Xie et al. (2016)
ICR mice	Measurement of enzymes such as superoxide dismutase, chloramphenicol acetyl transferase, glutathione 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Hydroxyl radical scavenging activity Superoxide anion radical scavenging activity	1.5 KDa	Qu and Han (2016)

Abbreviations: *DD* degree of deacetylation, *DP* degree of polymerization, *DPPH* 2,2-Diphenyl-1-picrylhydrazyl, *QCMCOS* quaternised carboxymethyl chitooligosaccharide, *ESR* Electron spin resonance, *DCFH-DA* Dichloro-dihydro-fluorescein-diacetate, *FRAP* ferric reducing power, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *CAT* Chloramphenicol acetyltransferase, *GSH-PX* Glutathione peroxidase, *NF-κB* Nuclear Factor kappa beta, *Nrf2* Nuclear factor (erythroid-derived 2)-like 2

activity while trimers and higher degree of polymerisation shows good anti-oxidant activity (Li et al. 2012). Partially acetylated version of chitotrimer was compared with deacetylated version and it was seen that acetylated chitosan has greater anti-oxidant activity proving that degree of acetylation has a role in anti-oxidant activity (Liaqat and Rengin 2018).

Chitooligosaccharides of five types were prepared using reactor where membrane of cut off 10, 5, 3 and 1 KDa was used for fractionation of various molecular weight chitooligosaccharide. Size of chitooligosaccharides varied according to pore size of membrane used. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging ability was found to be present in all types of chitooligosaccharide and mechanism involved pairing of odd electrons of the DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals. They also showed radical scavenging activities for all the free radicals but showed most effectiveness on DMPO (5,5-dimethyl-1-pyrroline N-oxide)-OH scavenging. Among all the fractions, those containing mol wt. 3–10 KDa showed

highest radical scavenging activity. In another study, radical scavenging ability of chitosan of various molecular weight (30, 90 and 120 KDa) was compared with that of an established synthetic anti-oxidant butylated hydroxytoluene (BHT) and an equivalent efficiency of 85% was obtained. Here also the lowest molecular weight chitosan showed highest activity (Hamed et al. 2016). Radical scavenging ability of chitooligosaccharides has also been studied in *in vivo* condition in a mouse model on high fat diet. Chitooligosaccharide showed potent anti-oxidant activity by protecting mice from oxidative stress (Qu and Han 2016) by reducing level of certain enzymes (Glutathione peroxidase, superoxide dismutase, catalase) in liver, serum and stomach which increases considerably in case of stress due to high fat diet. Gallic-acid conjugated chitooligosaccharides were been found to exert anti-inflammatory and anti-cancer effect on human lung epithelial cells (A549). It also depicted DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging and H_2O_2 induced DNA damage protection (Vo et al. 2017). Effects of chitooligosaccharide supplementation on performance, blood characteristics, relative organ weight, and meat quality in broiler chickens was analysed by Zhou et al. (2009). Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) are some of the reported synthetic anti-oxidants which are used in food industry to prevent oxidation and reduce rancidity and loss of flavours. However, because of health hazards related with these compounds natural and safe compounds are preferable and chitooligosaccharides has been found to be a suitable alternative of these compounds (Kim and Thomas 2006). Supplementation of chitooligosaccharide was reviewed on processing and storage quality of foods of animal and aquatic origin such as milk, meat, fish, eggs, sea foods, etc. (Singh 2016). Therefore, there is an increasing interest in antioxidants, particularly in those intended to arrest the presumed harmful effects of free radicals in the living systems, as well as the deterioration of fats and other constituents of foodstuffs (Rao et al. 2006).

3.3.2 Anti-Oxidant Activity of Chitooligosaccharides in Plants

Due to their strong antioxidant activity, chitooligosaccharide has been studied in plants for their agricultural application. Chitooligosaccharides at 1–10 mg/L with acetylation degree of 65% and molecular weight of 5–10 KDa significantly activated OPD (o-phenylenediamine) oxidation by wheat seedlings (Khairullin et al. 2001). Cabrera et al. (2006) reported effect of the degree of polymerization, degree of acetylation and concentration of chitooligosaccharide on defence activation in *Arabidopsis thaliana* suspension-cultured cells. Similarly chitooligosaccharides were able to induce nitric oxide (NO) generation followed by up-regulation in the activities of defence-related enzymes through an oligochitosan induced Ser/Thr protein kinase (OIPK)-dependent or independent pathway (Zhang et al. 2011). Plant

mineral nutrient dynamics was studied in hydroponically grown plants by Chatelain et al. (2014) and their use in phytoremediation and biofortification programs is promoted (Vasconcelos 2012). Application of chitooligosaccharide as a commercial preservative to improve the longevity of cut roses has also been studied (Jing and Li 2015). This was due to the decreased superoxide anion, hydrogen peroxide and malondialdehyde levels in the cut roses which protected them from withering.

3.4 Conclusion

In spite of making a lot of progress in the research area of "COS as an anti-oxidant" very few industries have used them in the area of food, pharmaceuticals and cosmetics. Their appearance as promising anti-oxidant biomolecules are being hindered by their non-availability on larger scale as obtaining chitooligosaccharide in highly purified form in bulk is still considered a difficult task. Downstream processing of enzymatically produced chitooligosaccharides is one of the key areas which is attracting the attention of researchers for getting purified fraction of chitooligosaccharide in terms of defined degree of polymerization and exact known sequence of monomer (both acetylated and deacetylated). More studies are required to establish relation between the anti-oxidant activity and degree of deacetylation/polymerisation and sequence in order to predict the connection between biological activity and their structure. Studies on *in vitro* and *in vivo* activity of chitooligosaccharides after characterisation are required for their establishment as an anti-oxidant molecule. However, most of these studies have been done on chitooligosaccharide mixtures which contained chitooligosaccharides molecule with various degree of polymerisation. Establishment of anti-oxidant activity due to any particular type of chitooligosaccharide was found to be difficult and needs to be explored further. Efficient biocatalytic production of chitooligosaccharides with good yield and cost effectiveness are desirable. Downstream processing studies leading to separation and purification of chitooligosaccharide with single degree of polymerization along with their *in vitro* and *in vivo* effect is another area where more investigation is needed. Apart from antioxidant activity, chitooligosaccharides has been implicated in medicinal uses due to their antimicrobial effect (Park et al. 2004), neuroprotective effect (Huang et al. 2015) and anti-tumour effect (El-Sayed et al. 2017). Recently nanoparticles of chitooligosaccharides and their conjugates have shown a promise as a drug delivery vehicle (Lu et al. 2015; Xu et al. 2016) and more research is needed for evaluating the anti-oxidant potential of nanoparticulate forms of chitooligosaccharide.

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Chapter 4
Enzyme Immobilization on Chitin and Chitosan-Based Supports for Biotechnological Applications

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Abstract Actual industrial enzymes have often high cost and instability. Such issues have restricted commercial application of such fragile biomolecules. Alternatively, immobilization of enzymes on suitable supports improves stability, cost-effectiveness and recyclability. Chitin and chitosan are ideal supporting material because they are biocompatible, biodegradable, plenty of reactive functional groups, non-toxic and cheap. Different derivatives of chitin support such as chitosan, chitosan film, chitosan nanoparticle, and chitosan nanocomposite has been used for enzyme immobilization. Chitosan-bound biomolecules display considerably improved biocatalytic potential as compared to native biomolecules. Chitosan immobilized enzymes have exceptionally high operational stability and reusability, and thus are suitable for industrial processing. This chapter reviews enzymes immobilized on chitin- and chitosan-based biomaterials, and applications to drug delivery and sustainable agriculture.

Keywords Enzymes · Immobilization · Stability · Reusability · Drug delivery · Sustainable agriculture

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4.1 Introduction

Chitin is the second most prominent biopolymer after cellulose found in nature (Rinaudo 2006). As per the new analytical research published, chitin market is expected to reach US$ 2900 million by 2027 (Future Market Insights 2017). It is obtained primarily from marine exoskeleton of crustaceans, namely shrimps and crabs. The most important derivative of this natural biopolymer is chitosan. It is derived from chitin by deacetylation process and composed of randomly distributed β-(1–4)-linked D-glucosamine (deacetylated) and N-acetyl-D-glucosamine (acetylated) units. Chitosan is soluble in acidic condition due to the free protonable amino groups present in the D-glucosamine units and available from low to high molecular weight. It is a polymer with high variability in its chemical and physical properties (Dutta et al. 2004; Wei and Qian 2008; Aranaz et al. 2009).

Chitosan form colloidal particles and entrap bioactive substances such as protein and nucleic acids through a number of mechanisms including chemical cross-linking, ionic cross-linking and ionic complexation. Chitosan has higher affinity to cell membrane that finds usage as a coating agent for liposome formulation as well as a transfection agent for non-viral gene delivery (Prabaharan and Mano 2005). The functional characterization of chitosan helps in understanding the biological properties such as biocompatibility, muco-adhesion, regenerative effect on connective gum tissue, accelerates the formation of osteoblast responsible for bone formation, fungistatic, spermicidal, antitumor, central nervous system depressant, immunoadjuvant, accelerates bone formation, wound healing, cell immobilization, emulsifying agent, permeation enhancing effect, anticholesterolemic and antimicrobial activity. Apart from these properties include specific applications such as drug delivery, tissue engineering, functional food, food preservative, biocatalyst immobilization, wastewater treatment, molecular imprinting and metal reductions (Dutta et al. 2004; Aranaz et al. 2009).

Chitosan finds its extensive application in various fields due to its low cost, large-scale availability, antimicrobial activity, biodegradability, non-toxicity and bioadhesive properties (Khor and Lim 2003). It also possesses special properties such as crossing of the blood brain barrier, as a haemostatic agent, fat attractor and further supporting its use in field bandage due to its natural antibacterial activity (Qi et al. 2004). Chitosan finds its applicability in variety of biomedical applications such as in human wound healing due to its availability as mesh membrane (Azad et al. 2004), and as an efficient gene delivery vehicle. It acts as DNA condensing agents due to the cationic polymeric property. It depends on a variety of factors such as complex size, complex stability, toxicity, immunogenicity, protection against DNase degradation, intracellular trafficking and processing of the DNA (Borchard 2001). Chitosan based matrices show interest particularly in tissue engineering for controlled drug release and tissue remodelling due to the fibrous and porous property (Bhattarai et al. 2005). Chitosan metal nanocomposites acts as a carrier for bioactive nanocarriers. Thus, it is used as regenerative medicine and drug delivery vesicles (Hein et al. 2008). A chitosan-based dietary food exhibits anticholesterolemic, antiulcer and anti-uricemic properties. It has high binding capacity specifically to

fatty acids, bile acids, phospholipids, uric acid and toxic gliadin fraction that lowers the production of putrefaction metabolites (Muzzarelli 1996).

Apart from chitosan biomedical application, it also finds its wide application in catalysis. It is available in the form of colloids, flakes, gel/hydrogels, beads, fibers which include hollow fibers, immobilized inorganic supports which uses alumina, silica, or other metal oxides to produce an efficient catalytic activity in reactions such as hydrogenation, oxidation, allylic substitution, polymerization, cyclopropanation of olefins, asymmetric dihydroxylation of olefins, carbonylation, monoglyceride synthesis and fine chemical synthesis (Guibal 2005). It also finds its application in water and wastewater treatment. Chitosan acts as an anion-exchanger in adsorbing the main acids components from the municipal, agricultural and industrial liquid or solid wastes which differ in their chemical, physical and biological characteristics (Lalov et al. 2000). It is a novel food preservative in food industry. Chitosan glucose complex, a modified form of chitosan, shows an excellent antioxidant property and antimicrobial activity (Kanatt et al. 2008). It is used as a dietary ingredient in weight-loss supplements. Chitosan shows binding and trapping of dietary fats that leads to fat excretion and weight loss without caloric restriction (Gades and Stern 2005).

The present chapter discusses the suitability of chitin and chitosan derived materials-based enzyme immobilization for various biotechnological applications such as drug targeting, industrial biotechnology, and sustainable agriculture.

4.2 Biomolecules Immobilization Using Chitin Derived Supports

During the last few decades, the application of enzymes biomolecules in different industries is continuously increasing. Global market for industrial enzymes was $4.2 billion in 2014. It is expected to expand with a compound annual growth rate of approximately 7% to reach approximately $6.2 billion over the period from 2015 to 2020 (Singh et al. 2016).

Enzymes are intimately involved in an extensive variety of out-dated food processes, include food such as baking, dairy products, starch conversion and beverage processing such as beer, wine, fruit and vegetable juices, animal feed, textiles, pulp and paper, detergents, biosensors, cosmetics, health care and nutrition, wastewater treatment, pharmaceuticals and chemical manufacture and, more recently, biofuels such as biodiesel and bio-ethanol (Kjeang et al. 2006; Janaun and Ellis 2010; Parawira and Tekere 2011; Verma et al. 2009, 2011, 2012, 2013a, b, c, 2016).

Thriftily sustainable and an extremely stable form of enzyme is a prerequisite in terms of their reusability for the Industrial applications of enzymes. Because of their high stereo-, chemo-, and regio-selectivity, the attention was nurtured towards biocatalysts for chemical manufacturing. Chemical reactions are catalyzed by the proteins called as enzymes. Enzyme lowers the activation energy of the reaction that enables the conversion of substrates into products by providing favourable conditions. An enzyme consists of at least one polypeptide moiety and they might be a

glycoprotein or a protein (Karigar and Rao 2011). Enzymes are huge and friable molecules contrasting to the customary inorganic and organic catalysts. However, enzymes provide a competent and eco-friendly catalyst without a prerequisite of high temperatures, pressures, and harsh chemical surroundings. Nevertheless, the practical application of enzymes is limited even with the advantages. Whereas the enzyme shows effective catalytic activity under mild circumstances, that is, at aqueous media and ambient temperatures, the stability and activity are also restricted to operation under those circumstances. The disadvantages of using soluble enzymes are their instability and sensitivity to process conditions. For industrial production, the high cost, fragile nature, and high loading requirements restricted the applicability of soluble enzyme. Hence, usage of solid phase biocatalysts has grasped the attention of researchers during last decades (Cao et al. 2001; Sheldon et al. 2007; Biro et al. 2008).

Enzyme immobilization is an exciting alternative to overcome the high-cost and instability problems of the biocatalysts at the industrial level (Lopez et al. 1997; Carrea and Riva 2000; Cao et al. 2003; Sheldon 2011). An immobilized enzyme is therefore an enzyme which is attached to an inert, organic or inorganic, insoluble material such as calcium alginate and silica. Furthermore, the attachment of an enzyme to a solid support can increase its resistance to various environmental changes such as pH or temperature (Dalal et al. 2007; Kanwar et al. 2008; Kanwar and Verma 2010; Homaei et al. 2013). Immobilization plays an important key role in improving the operational performance of an enzyme in industrial methods which are primarily use in non-aqueous media. Various methods for the immobilization of enzymes are critically studied. The methods are divided into three main categories (a) binding to a prefabricated carrier (b) entrapment in organic or inorganic polymer matrices, and (c) cross-linking of enzyme molecules (Cao et al. 2003; Kanwar et al. 2005, 2006, 2007a, b; Sheldon et al. 2007). In recent developments, for the use of novel supports for enzyme immobilization, importance has been given e.g. mesoporous silicas, hydrogels, polymers, novel entrapment methods such as cross-linked enzyme aggregates. So, for the process of enzyme immobilization, many different types of organic and inorganic carriers such as alginate beads, polyaniline matrix, glass beads, microporous polypropylene hollow fiber membranes, activated carbon, chitosan film, modified polyvinylidene fluoride microfiltration membrane and magnetic chitosan microsphere has been used (Domínguez et al. 2007).

The most of the industrial applications of immobilized enzymes demonstrate reusability, improved stability, and exhibit higher potential application than their free forms. Immobilized enzymes have huge prominence in industrial bioprocesses exclusively in food, pharmaceutical and nutritional technologies (Sheldon 2007). There are numerous reasons for using an enzyme in an immobilized form, in addition to more appropriate handling of the enzyme, it also provides a facile isolation from the product. The purification costs reduce as it assists in preventing the impurity of the substrate with other compounds or enzyme/protein (Spahn and Minteer 2008). The effective retrieval and reuse of costly enzymes, with higher half-lives and fewer degradation was accomplished when immobilization was accounted. The bioprocess can ultimately be carried out in a continuously working reactor in consecutive batches (Shi et al. 2011). The properties of their carriers, such as material

types, structures, and compositions plays a vital role in deciding the catalytic behaviour of immobilized enzymes.

The chemical property of chitosan includes linear polyamine, reactive amino groups and reactive hydroxyl groups available and chelates many transitional metal ions. As chitosan contains many amino groups (polycations), it stabilizes the nanocarriers supports and provides surface modification which finds its applicability as a carrier in biotechnological field. The reactivity of primary amino groups and primary and secondary hydroxyl group help to finds its application in diversified fields (Dutta et al. 2004; Wei and Qian 2008; Aranaz et al. 2009). The characteristic properties such as biocompatibility, biodegradability and non-toxicity possessed by it make as a potential candidate for conventional and novel drug delivery systems. Chitin and its derivative chitosan have certain incredible properties that qualifies them as good supports for enzyme immobilization. They respectively carry one acetylated or primary amino group on each anhydroglucose unit: the degree of acetylation (approximately 82% for chitin and approximately 20% for chitosan) as well as the molecular weight can be accurately calculated. The amino groups can assist in attachment of bridge molecules like glutaraldehyde when trying to form covalent bonds with a protein. In fact, the above cited studies make use of glutaraldehyde for the immobilization of the enzymes on chitin or take advantage of the chelating ability of the polymers with metal ions to provide intermediate species with which the protein would interact. Chitin has been used as a solid support to immobilize many enzymes acid phosphatase, chymotrypsin, glucose isomerase, galactosidase, glucose oxidase, and lactase (Muzzarelli et al. 1976).

Immobilized enzymes are more strongly resistant to environmental changes compared to free enzymes (Dalal et al. 2007; Verma and Kanwar 2008, 2010, 2012; Garcia-Galan et al. 2011). More prominently, the heterogeneity of the immobilized enzyme systems allows an easier recovery of both enzymes and products, several times reusability of enzymes and continuous operation of enzymatic processes (Sheldon et al. 2007; Verma et al. 2008a, b, 2017a, b, c; Garcia-Galan et al. 2011).

It is inferred from the above-cited studies that immobilization of biomolecules, in particular to biocatalyst, on the chitin/chitosan supports has improved enzyme properties for the potential industrial applications. Furthermore, chitin/chitosan supports based enzyme immobilization studies have prominently demonstrated broader applications ranging from biomedical to sustainable agriculture sectors. The contributions of chitin/chitosan based enzyme immobilization for various biotechnological applications is discussed in next three sections.

4.3 Enzyme Immobilized Chitin/Chitosan Support for Biomedical Applications Including Drug Targeting

Initially, chitosan and chitin were reported to contain therapeutic properties such as antimicrobial (Tsai et al. 2002), analgesics (Konno et al. 2002; Okamoto et al. 2002), wound healing (Ohshima et al. 1987), cartilage tissue engineering (Suh and Matthew 2000), growth factor (Howling et al. 2001), blood coagulation (Okamoto

et al. 2003), and antifungal (Stossel and Leuba 1984). But its other properties suiting as excipient for pharmaceutical application overweighed the pharmacological activities. Thus, it was a more suitable biomaterial for researcher active in pharmaceutics, drug formulations and drug delivery system. This biomaterial has been suitable as drug delivery excipient not only for small molecules but also for macromolecules like proteins, enzymes, growth factors, nucleic acids (RNA, DNA/genes) etc. Chitin and chitosan are unstable at gastric pH due to amino acid protonation. Thus, presystemic metabolism as utmost drawback has been reported. However, many other functionalized chitosans such as carboxylated, thiolated and other conjugated chitosan have been successfully explored to overcome the drawback of presystemic metabolism.

Uncountable formulations of chitosan as excipient are already there in the market. Apart from this inexhaustible research on drug delivery system taking chitosan as excipient/carrier in recent years and still a hot research topic reveals its perfect suitability. Following are chitosan properties which makes it suitable for drug delivery system (Elgadir et al. 2015):

1. Anionic drug delivery properties
2. Mucoadhesive properties/Gelling properties
3. pH responsive
4. Infra red responsive
5. Gene expression properties

Few recent and/or breakthrough studies representatives of each class are given in Table 4.1 which shows chitosan effective approach in wide range of properties. Cationic property of the chitosan makes it most suitable material for delivery of anionic drugs. Mucoadhesive property is again due to its cationic charge. There are many other alternatives for this property but the collective delivery favoring properties makes it suitable material even for mucoadhesive formulations. Moreover, this property can also be modulated by copolymerization or functionalization. Chitosan with branches has been reported to enhance the gene transfer properties and few other cases has also been mentioned in Table 4.1. Again, positive charge on chitosan has been attributed to its permeation enhancing property because of its ability to restructuring the membrane proteins or tissue cementing materials. Apart from drug delivery properties, chitin or chitosan has also been appropriate for enzyme immobilization due its high affinity to proteins, functional groups reacting with enzyme's functional groups or with other functionalization agent, easy and variable configurational synthesis and control at various step along with availability of large literature for estimation of feasibility of researcher's vision.

Many research studies on enzyme/protein immobilization has been carried out for biomedical applications using chitin/chitosan supports. Plethora of research are going on for possibilities of chitosan compatibility with enzymes/proteins for different applications (Table 4.2). Here we are specifically concerned with drug targeting and biomedical application like biosensor, and dialysis. Most of the studies just checked the compatibility of enzyme or protein immobilized but few studies evaluated the preparation/formulation for suggested application. Many novel strategies are

Table 4.1 Properties of chitosan exploited in recent research for various modes of drug delivery systems. Unique property of chitosan such as anionic, mucoadhesive, infrared, pH, magnetic, and permeation enhancing property etc. are being exploited for drug delivery

Property of chitosan exploited	Drug delivered	Specifications	References
Anionic drug delivery	Diclofenac sodium	Chitosan in montmorillonite along with alginate	Kevadiya et al. (2015)
Anionic drug delivery	Methotrexate	Chitosan nanosphere	Dhanaraj et al. (2016)
Anionic drug delivery	5-Fluorouracil	Cyclodextrin/alginate chitosan nanoflowers	Lakkakula et al. (2017)
Anionic drug delivery	5-Amino-salicylic acid	Pectin coated chitosan/layered double: Colon targeting	Ribeiro et al. (2014)
pH sensitive and delivery of anionic drugs	Ciprofloxacin	Chitosan coated iron oxide nanoparticles. Enhanced drug release kinetics by low-frequency ultrasounds	Kariminia et al. (2016)
pH sensitive release	Doxorubicin	Magnetic chitosan nanoparticles	Unsoy et al. (2014)
Magnetic and pH sensitive release	Riboflavin	κ-Carrageenan and carboxymethyl chitosan beads with *in situ* synthesized magnetic nanoparticles	Mahdavinia et al. (2015)
Infrared and pH sensitive	Doxorubicin	Chitosan + single-wall carbon nanotubes encapsulated in nanogel + poly ethylene glycol diacrylate	Qin et al. (2015)
pH sensitive	Insulin	Chitosan-alginate nanoparticles for oral delivery	Mukhopadhyay et al. (2015)
Mucoadhesive	Sulfasalazine	Catechol functionalized, genipin crosslinked-chitosan gel for buccal targeting	Xu et al. (2015)
Mucoadhesive	Metoclopramide hydrochloride	5-Methyl-pyrrolidinone chitosan nasal delivery	Gavini et al. (2008)
Gene expression	Peanut allergen gene for immunization against peanut allergy	Chitosan nanoparticles	Roy et al. (1999)
Gene expression	Small interfering RNA for permeability-glycoprotein	Glycol chitosan nanoparticles	Yhee et al. (2015)
Permeation enhancing property	Serratiopeptidase	Chitosan coated magnetic nanoparticles	Kumar et al. (2014b)

being explored in last decades to targeting the drugs taking chitosan or hybrid chitosan as a carrier molecule but there are very few instances where therapeutic enzymes or biologically active protein immobilized/encapsulated/conjugated with chitosan at

Table 4.2 Chitin or chitosan immobilized enzyme employed for biomedical applications including drug delivery. Chitosan possess multifunctional group that are being exploited to immobilize enzyme or other biomolecules using a suitable cross-linker or other specific interaction

Enzyme	Immobilization matrix	Application	References
Serratiopeptidase	Chitosan-coated magnetic nanoparticles	Drug targeting: Permeation enhancer and anti-inflammatory agent	Kumar et al. (2014b)
Tissue plasminogen factor	Chitosan-coated magnetic nanoparticle	Drug targeting: Thrombo-embolism	Chen et al. (2011)
Insulin	Trypsin inhibitor and goblet cell targeting ligand attached chitosan nanoparticles preparation	Trypsin resistant insulin preparation for oral insulin delivery	Chen et al. (2015)
Proneural rat interferon-γ biotinylated with avidity tag	Thiolated methacrylamide chitosan	Neural cell differentiation scaffold	Leipzig et al. (2011)
Antibodies against intercellular adhesion molecule-1 in intestine	Chitosan: Alginate (shell: Core) microcapsules	Oral drug delivery formulation	Ghaffarian et al. (2016)
Glucose oxidase	Chitosan mat	Antibacterial preparation by constant production of H_2O_2 as oxidizing agent	Bosiger et al. (2018)
Lactate dehydrogenase	Composite film of multiwalled carbon nanotubes-chitosan	Lactate biosensor	Tsai et al. (2007)
Urease	Chitosan beads	Immobilized urease for urea analysis in samples, equivalent to autoanalyzer and potential in hemodialysis	Kumar et al. (2009)
Trypsin	Maghemite magnetic nanoparticles functionalized with chitosan	For protein degradation for enhanced detection in mass spectrometry	Kluchova et al. (2009)
Urease	Hydrogel of chitosan–alginate complex with poly(acrylamide-co-acrylic acid)/k-carrageenan hydrogels	Urease immobilized chitosan-based hydrogel for potential application in hemodialysis and blood urea detoxification	Kara et al. (2006)
Oxalate oxidase-catalase	Chitosan	Potential for hyperoxaluria	Ramakrishnan et al. (1997)
Alcohol dehydrogenase	Composite film of multiwalled carbon nanotubes bound with chitosan	Biosensor for ethanol	Lee and Tsai (2009)
Glucose oxidase	Sandwich configuration: Chitosan-ferrocene: Glucose oxidase: Chitosan	Blood glucose biosensor	Miao et al. (2001)
Oxalate oxidase	Mucin/chitosan gel crosslinked with glutaraldehyde	Amperometric biosensor for oxalate determination	Benavidez et al. (2009)

any form (gel, micro/nano particles, films etc. or hybrid with other polymer carrier matrix). Nano form of chitosan have been found to more effective as nanocarrier due to nano size, high surface area per unit volume/weight, controlled drug release in response to specific stimuli, inherent pharmacokinetic and pharmacodynamic properties. Chitosan nanoparticles itself, is inhibitor of permeability-glycoprotein (Saneja et al. 2014), spontaneously engulfed by microfold cells of intestine, have the ability to bypass the proteinaceous cementing materials of tight junctions among the adhering cells thus enhances the cellular permeability (Dutta 2016).

Among three different studies on enzyme serratiopeptidase immobilization on magnetic nanoparticles with different functionalization agents, functionalization of magnetic nanoparticles with chitosan in combination with glutaraldehyde as spacer (Fig. 4.1) was found to be suitable carrier with respect to 535 μmols g^{-1} magnetic

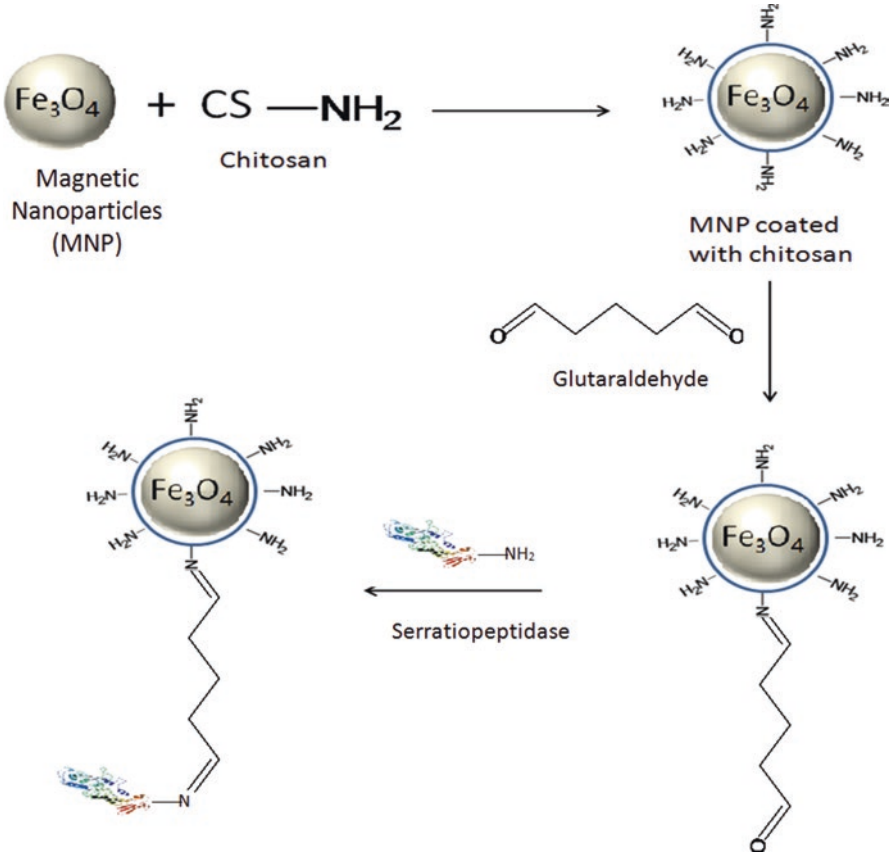

Fig. 4.1 Immobilization of enzyme serratiopeptidase on chitosan coated magnetic nanoparticles. Magnetic nanoparticle was surface-functionalized with chitosan. The functionalized magnetic nanoparticle having amino group is covalently bound with the amino group of the serratiopeptidase enzyme *via* a glutaraldehyde cross-linker, thus making a robust chitosan coated magnetic nanoparticle immobilized enzyme system

nanoparticles surface amino groups, protein loading (264 mg g^{-1} magnetic nanoparticles), enzyme loading (325 U g^{-1} magnetic nanoparticles) and 52 enzyme molecules per magnetic nanoparticles. Enzyme serratiopeptidase has membrane permeation enhancement activity so its combination with chitosan was expected to potentiate the enzyme's effect as per chitosan action on membrane. The permeation enhancement study of this chitosan coated magnetic nanoparticles preparation of enzyme was carried out with modified Franz's diffusion cell. Apart from comparison among the preparation, even with thickest coating, *in vivo* studies on rat model of inflammation showed the significant effect of magnet application on inflamed rat paw. Here the formulation research may get the biocompatibility of chitosan along with insignificant loss in magnetic property due to its coating (Kumar et al. 2013, 2014a, b).

Another chitosan coated magnetic nanoparticles immobilizing a clot dissolving protein/enzyme i.e. tissue plasminogen activator was a success story of magnetic targeting where chitosan probably has contributed. For this e*x-vivo/in-vitro* models have been developed to assess the magnetic capability along with enzyme or protein activity retention. One of such intravascular thrombolysis model was driven by a constant pressure gradient, similar to the condition that maintained blood circulation *in vivo*, was used to study blood clot lysis by tissue plasminogen activator or tissue plasminogen activator-chitosan-magnetic nanoparticle. Tissue plasminogen activator-chitosan-magnetic nanoparticle under magnetic guidance can reduce the blood clot lysis time by 58% compared to the same without magnetic targeting or by 53% compared to free tissue plasminogen activator using the same dosage of the thrombolytic drug i.e. 0.1 mg tissue plasminogen activator mL^{-1} (Chen et al. 2011).

An interesting study with objective of oral delivery of insulin in conjugated nanoparticles of poly(g-glutamic acid) and trimethyl chitosan modified with L-phenylalanine adopting multi ion crosslinked method for more stability. Initially poly (g-glutamic acid) was attached with active trypsin inhibitor Camostat mesylate derivative for confirming the insulin protection from the intestinal protease while chitosan was attached with goblet cell targeting peptide for targeted intake of the nanoformulation. Fluorescein isothiocyanate was also attached with this formulation for probing. This nanoformulation was evaluated for transmembrane permeation using Ussing chamber and was reported to have significantly high cumulative permeation of amount of drug in comparison to different controls (Chen et al. 2015).

Neural stem cells can be differentiated to neurons and has the potential to treat many neurodegenerative diseases, injury to central and peripheral nervous system, and stroke. While interferon-γ is a growth factor of cytokine class that signals neural stem cells to differentiate to neurons. A study immobilized proteinaceous interferon-γ to methacrylamide chitosan hydrogel to provide the progenitor cells a 3-dimensional structure with growth factor for differentiation. Recombinant interferon-γ was taken, containing biotin attached at its one end while methacrylamide chitosan was attached with streptavidin for well-known strong affinity binding with each other (streptavidin-biotin). The methacrylamide chitosan was also attached with cell adhesive peptides for providing binding platform for naïve progenitor/undifferentiated cells (Benavidez et al. 2009).

Other recent representative examples of biomedical, biosensing and analytical applications of the different forms of chitosan attached with different enzymes or

proteins have been summarized in Table 4.2. Initially chitin, now it's more beneficial deacetylated derivative chitosan has been of great importance to formulation scientists due to its reproducible and controllable properties required for drug delivery. This polymer is also suitable for all types of immobilization of enzymes or proteins due to its functional group present (covalent immobilization), due to its charge (electrostatic attraction/adsorption), and its ability to form a matrix (entrapment/encapsulation). This biomaterial is of choice specially if the enzyme or protein or small molecule drug is of therapeutic in nature due to its non-toxic biocompatible nature.

The above cited studies conclude the involvement of chitosan in successful strategies and ideas related to drug delivery to be pavement to possibility of novel ideas for upcoming researchers.

4.4 Chitin/Chitosan Immobilized Enzyme in Industrial Biotechnology

This section explains the immobilization of various enzymes on chitin and chitosan as a carrier/support material for industrial biotechnology applications. The selection of the suitable carrier is another tough task in immobilization process.

A vital industrial enzyme is used for manufacturing synthetic cephalosporins and penicillins, the most widely used antibiotic, is Penicillin G acylase. It catalyses the deacylation of penicillin G to produce 6-aminopenicillanic acid, an important intermediate in the manufacture of β-lactam antibiotics (Bianchi et al. 1996). Additionally, industrial processes are targeted on the condensation of the suitable D-amino acid derivative with the β-lactam ring catalysed by penicillin G acylase for the comprehensive production of synthetic antibiotics (Arroyo et al. 2003). One-point and multipoint covalent attachment to the solid matrix are the two dissimilar immobilization approaches which was studied. Pragmatically, immobilization yield was 82% in the multipoint covalent attachment derivative. At 50 °C, stability was 4.9-fold more than the free enzyme and at pH 10.0, 4.5 times more stable than the soluble enzyme. While, in case of one-point derivative immobilization yield was 85%. But in this case also, 2.7-fold more stability was observed than the free enzyme at 50 °C and 3.8-fold more stable than soluble Penicillin G acylase at pH 10.0. Chitosan can be mixed with Penicillin G acylase above 330 IU/g as inferred from the results. However, at 37 °C and 25 °C, the intraparticle diffusive effects limited the hydrolysis of penicillin G catalyzed by those derivatives. The multipoint derivative exhibited a half-life of 40 hours when assays for operational stability were performed (Adriano et al. 2005).

β-glucosidase is acknowledged as an effective catalytic agent for the hydrolysis of several glycosides commonly occurs in numerous forms of living creatures (Njokweni et al. 2012). Lately, it was stated to be used for hydrolysis of cellulose (Shewale 1982), improvement of aroma in wine (Palmeri and Spagna 2007), tea (Su et al. 2010), bioconversion of isoflavone glycosides to aglycones (Rekha and Vijayalakshmi 2010), and fragrance in fruit (Fan et al. 2011). For their impending industrial applications, this enzyme is currently getting considerable attention.

Nevertheless, there are few limitations for wide usage of β-glucosidase, because of instable structure and the extraordinary price of enzyme recovery for continual applications (Nisha et al. 2012; Dicosimo et al. 2013; Sheldon and van Pelt 2013). So, immobilization is a way to overcome the drawbacks associated with the application of β-glucosidase. It has been observed that enzyme concentration and adsorption time were the factors which significantly influences, and from response surface methodology, the maximum activity recovery up to 50.75% was obtained which can be exceptionally correlated with the experimental value of 50.81%. Moreover, several properties of immobilized β-glucosidase were estimated. Compared to the free β-glucosidase, the immobilized enzyme displayed broader temperature and pH ranges, better storage stability, improved thermal stability, and reusability and higher accessibility of the substrate to the immobilized β-glucosidase (Zhou et al. 2013).

Cellulase and xylanase are extensively used in the biotechnology trade in its immobilized form, among other things for the bioconversion of agricultural waste, for extracting plant oils and coffee, for improving the digestibility of animal feed ingredients and for clarifying juices and wines (Butt et al. 2008). Presently, they are also employed in the bioconversion or biodegradation of materials containing cellulose and hemicellulose to monomeric sugars (Liu et al. 2006). Researchers have reported that agricultural waste having high amount of lignocellulosic content could be used for the production of a whole range of marketable products comprising organic acids (Shen and Xia 2006), ethanol (Qu et al. 2006), and, other chemical products (Cao et al. 1997) also if the practice were economically inexpensive. Two commercial enzymes namely cellulase and xylanase were immobilized using three chemical methods such as adsorption, reticulation, and crosslinking-adsorption. Alginate-chitin and chitosan-chitin were the two polymeric supports used for immobilization. Researchers observed 4.5 pH as optimal for binding for cellulase and 5.0 pH for xylanase, and the ideal enzyme concentrations were 170 μg/mL and 127.5 μg/mL, respectively. The chitosan exhibited the property of an ideal support. A small amount of glutaraldehyde, a cross-linking agent enhanced the stability of the immobilization method in some of the cases. The reusability of enzymes was the most notable outcome witnessed by biotechnological characterization, predominantly of immobilized cellulase using glutaraldehyde, which retained 64% activity even after 19 cycles. The results were an addition in confirming the profitable and biotechnical rewards of enzyme immobilization for a series of industrial usage (Romo-Sánchez et al. 2014).

Valerio et al. (2013) evaluated ionotropic gelation method and activation with glutaraldehyde for the preparation of chitosan nanoparticles. The prepared nanoparticles were evaluated for immobilizing invertase enzyme from *Saccharomyces cerevisiae*. This enzyme has great role in sucrose hydrolysis, humectant agent for candies production, preparation of artificial honey, besides other applications as in drug, cosmetic, and paper industry (Kotwal and Shankar 2009).

During biofuel production, one of the predominant problems is yeast flocculation (*Saccharomyces cerevisiae*). Operational difficulties were observed which ultimately enhances the ethanol cost. This difficultly can be resolved using proteolytic enzymes since it does not depend on fluctuations as reported by Silva et al. (2015). This group of researchers focused their study on recycling of soluble papain enzyme

and its immobilization on chitin or chitosan. Three cross-linking agents were assessed in the action of proteolytic activity of papain. The polyethyleneimine, glutaraldehyde, and tripolyphosphate deactivated the enzyme in this range, respectively. While, glutaraldehyde showed inhibition of papain immobilization in its all treatments. After a reaction time of 5 h, 15.7% and 6.07% yield of active immobilized enzyme was observed in case of chitosan cross-linked with tripolyphosphate and chitosan treated with 0.1% polyethyleneimine, respectively. Though small amount of active enzyme immobilization was achieved, but such levels is not sufficient to inhibit flocculation of yeast cells. The study observed that free enzyme was competent for yeast deflocculation in dosages of 3–4 g L^{-1}. Up to 14 cycles, the recycling by centrifugation of soluble papain was effective with yeast suspension which is perfectly compatible to industrial conditions (Silva et al. 2015).

One of the most important groups of industrial enzymes that are widely used in detergent, leather and meat industries are the serine alkaline proteases. Such proteases occupy approximately 35% of the microbial enzyme sales (Biswanath et al. 2012). Peinado et al. (2006) reported various types of immobilization procedures and their extensive applications in the area of biotechnology, environmental, food, pharmaceutical, and biosensor industries. Immobilization of protease enzyme onto suitable support materials plays a crucial part in several fields of technology comprising the detergent and food industries. Therefore, due to its applications in biocatalysis, the progress in the methodologies of protease immobilization has been extremely regarded. Process of enzyme immobilization involves suitable carrier/support, so the natural polysaccharides, alginate and chitosan have been studied extensively. Satisfactory activity and stability were observed when proteases were physically immobilized in alginate-chitosan beads. The beads were produced by dropwise adding of protease-alginate mixture to the chitosan and calcium chloride solution. Then immobilized proteases were encapsulated in alginate-chitosan beads. Various variables such as pH, temperature, and stability of the enzyme were studied. Optimum temperature of 47 °C and pH 8.5 was observed for immobilized protease. Rezakhani et al. (2015) demonstrated that the protease enzyme immobilized in alginate-chitosan beads displayed sensibly good activity and stability (Homaei 2015; Mohamad et al. 2015).

For bioethanol production, cellulose and hemicellulose obtained from municipal and agricultural waste are hydrolyzed to fermentable reducing sugars, and then, the ethanol is produced by fermenting the sugar. The enzymatic hydrolysis of lignocellulosic materials to produce glucose is being smoothened by the usage of cellulase enzyme complex which comprises three components of enzyme (Sun and Cheng 2002). Because of hydrophilic nature, there are narrow practical applications of cellulase enzyme. Zang et al. (2014) reported that chitosan coted magnetic iron nanoparticles facilitated cellulose enzyme immobilization onto them. At varied temperature and pH conditions, the immobilized enzyme showed higher operational stability than the free enzyme.

Another study conducted by Díaz-Hernández et al. (2018) reported that the application of genetic engineering techniques is required for the industrial enzymes of commercial value. The authors used new single step alkaline precipitation method

for the synthesis of chitosan coated magnetic iron nanoparticles to maximize protein loading. Chitosan is known as natural and low toxic cross-linker agent. High protein loading and more magnetic strength providing protocols are needed for industrial application, so as to minimize the operational cost.

Chitosan nanoparticles are natural constituents with eco-friendly material. Such nanoparticle possess bioactivity that does not harm humans having excellent physico-chemical, antimicrobial and biological properties, which make them a superior choice amongst scientists. Chitosan nanoparticles has wide application due to distinctive properties, ranging from tissue engineering, pharmaceutical, and food packaging to biosensing, waste water treatment and enzymes immobilization.

Thus, it can be inferred from the above discussed studies that versatile chitin derived supports employed for various forms of industrial and theraputic enzymes immobilisation provides an excellent platform to achieve high yield bioprocess.

4.5 Chitin Derived Supports Immobilized Enzyme in Sustainable Agriculture

The immobilization of various types of enzymes such as oxidoreductases, hydrolases and lignolytic enzymes on chitin and chitosan as a carrier/support material and their potential applications for sustainable agriculture including agri-food industry and environment biotechnology is discussed in this section.

Chitosan is the underutilized biopolymer that has vast potential to be explored in the near future especially in environmentally-friendly applications in systems working in natural environments, among others as enzyme immobilization supports. The enzymatic technology in industrial practice has been increased due to current demands of sustainable demands. Employment of enzyme as biocatalysts offers the benefits of mild reaction conditions, biodegradability and catalytic efficiency (Krajewska 2004).

Recently, the various enzymes such as oxidoreductases and hydrolases used in agri-food industries which are immobilized on chitosan as a carrier/support material has been summarized (Table 4.3). Enzymes like pectinases and cellulases, invertases, alcohol dehydrogenase, pepsin, amylases and α-amylase are immobilized on suitable carrier or matrices and are successfully used in the commercial production of jams, jellies, and syrups from fruits and vegetables. e.g. cellulose fibers were used to immobilized lactase for the production of lactose free milk from milk. Tripathi et al. (2007) and Lei and Bi (2007) immobilized the α-amylase and pectinase enzyme on chitosan beads and found the thermal stability of enzymes and the ease of its immobilization on low-cost matrices and good stability after immobilization. Abdel-Naby et al. (1998) studied the immobilization of alkaline protease and optimal reaction temperature of the immobilized enzyme was increased from 50 to 55 °C. Further, the thermal and storage stabilities of the enzyme were significantly improved after immobilization process.

Table 4.3 Chitin or chitosan immobilized enzyme used in agri-food industry. Plethora of agriculture-food industry application is being achieved using chitosan immobilized enzymes. Enzymes of class oxidoreductase and hydrolases are mostly commonly employed for agri-food industry applications

Enzyme	Support	Application	References
Aminoacylase	Chitosan-coated alginate beads	Production of L-phenylalanine	Lee et al. (1992)
Alanine dehydrogenase	Chitosan beads	Determination of aliphatic amino acids	Kiba et al. (1993)
Alkaline phosphatase	Chitosan beads	Used as a marker for milk pasteurization in dairy industries	Agarwal and Gupta (1995)
Alkaline protease	Chitosan powder	Used in leather, detergents and textile industries	Abdel-Naby et al. (1998)
Invertase	Chitosan powder and solution	Used in bakery	Hsieh et al. (2000)
Alcohol dehydrogenase	Chitosan beads	Used in food, pharmaceutical and fine chemicals industries	Soni et al. (2001)
Alcohol oxidase	Chitosan beads	Determination of ethanol in liquor samples	Taniai et al. (2001)
Acid phosphatase	Chitosan beads	In protein labeling, dephosphorylation of nucleic acids and for making enzyme based biosensors	Kurita (2001)
β-Amylase	Chitosan beads	Production of maltose	Noda et al. (2001)
Cellulase	Chitosan beads	Biofuel production, food and feed industry and brewing	Darias and Villalonga (2001)
Lipase	Chitosan flakes and beads	Detergent, food and flavor industry	Pereira et al. (2001)
Papain	Chitosan beads	Used in meat tenderization, foods, feeds, brewing and the textile industry	Li et al. (2010)
Uricase	Chitosan membrane	Enzymatic oxidation of uric acid	Yao et al. (2003)
Laccase	Magnetic chitosan microspheres, chitosan nanoparticles	Brewing, color enhancement in tea, cork modification, wine and beer stabilization, fruit juice processing	Jiang et al. (2005) and Fang et al. (2009)
α-Amylase	Chitosan beads	Used in food, fermentation and pharmaceutical industries	Tripathi et al. (2007)
Pectinase	Chitosan beads	Production of good quality paper and fermentation of coffee and tea	Lei and Bi (2007)
Horseradish peroxidase	Chitosan membrane	Removal of hydroxylated aromatic compounds	Monier et al. (2010)
Glucoamylase	Fe_3O_4–chitosan nanoparticles	Use in the saccharification of partially processed starch/dextrin to glucose	Wang et al. (2012)
Pepsin	Chitosan beads	Modify and provide whipping qualities to soy protein and gelatin, vegetable proteins for use in nondairy snack items, precooked cereals into instant hot cereal	Altun and Cetinus (2007)

Likewise, Hsieh et al. (2000) reported immobilization of invertase using chitosan as a carrier for covalent coupling, which improved the activity and thermal stability of immobilized invertase. Furthermore, immobilization of invertase on chitosan supports triggered the optimal reaction pH to shift from 4.5 to 2.5. Jiang et al. (2005) and Fang et al. (2009) reported the immobilization of the laccase enzyme on magnetic chitosan microspheres and chitosan nanoparticles respectively and showed thermal, operational and storage stabilities of the laccase enzyme were improved significantly after they were immobilized on the surface of the magnetic chitosan microspheres. Cellulase, the most important enzyme, is used for the production of biofuel used in the conversion of cellulose into glucose. Darias and Villalonga (2001) studied the linking of cellulose and chitosan by covalent conjugation and found that thermostability was increased by 8.9 °C for the cellulase-chitosan complex and stability within the pH range 1.0–3.2 was also improved.

Recently, many studies of environment remediation have been carried out using chitosan immobilized enzymes (Shao et al. 2007; Dinçer and Telefoncu 2012). In treatment of sewage and industrial effluents using immobilized enzymes such as laccase, lignin peroxidase, manganese peroxidase collectively known as lignolytic enzymes, polyphenol oxidase, chloro-peroxidase, parathion hydrolase and tyrosinase on chitosan and chitin has been summarized in Table 4.4. In case of laccase enzyme, many researchers have reported that by immobilizing enzymes enhanced the thermal stability and degradation of various dyes. It has also been reported that generally glutaraldehyde is chosen for the formation of cross-linked enzyme aggregates, but glutaraldehyde presents certain adverse environmental issues so to overcome the issues, an alternate has been found to cross-link the aggregated enzymes destined for environmental processes, renewable biopolymer chitosan has replaced glutaraldehyde. Shao et al. (2007) and Dinçer et al. (2012) reported that immobilization of polyphenol oxidase and tyrosinase on chitosan-SiO_2 gel and chitosan clay enhanced the removal of phenol from the aqueous media, respectively. Likewise, Bilal et al. (2016) and Sofia et al. (2016) reported the improved decolourization of textile effluent by the immobilization of enzyme manganese peroxidase and lignin peroxidase on chitosan beads respectively.

Nanomaterials can provide a promising hold as a carrier material, due to its smaller size and high specific surface area for loading of large number of amino groups, chemically modifiable surfaces and easier preparation (Krukemeyer et al. 2012; Verma and Barrow 2015; Verma et al. 2016). Iron oxide nanoparticles have shown a considerable potential in immobilization studies due its chemical inertness, biocompatibility and superparamagnetism. A particle is expected to show its magnetic property when exposed to a magnetic field. But magnetic nanoparticles with size lesser than 30 nm size show superparamagnetism and hence can be separated easily from the solutions using a strong magnet (Darwish et al. 2015). The biocompatibility of chitosan is good enough to be used in magnetic-field assisted drug delivery, enzyme or cell immobilization and many other industrial applications. Recently, nanotechnology provided different forms of nanomaterials that has enormous potential in the area of preparation of nanocarriers immobilized enzymes. This study explored at characteristics and applications of chitosan and chitosan

Table 4.4 Chitin or chitosan immobilized enzyme used in environmental remediation. Specific group of enzymes such as laccase, polyphenol oxidase, and peroxidase etc. are being employed for bioremediation applications

Enzyme	Support	Applications	References
Polyphenol oxidase	Chitosan-SiO_2 gel, chitosan-coated polysulphone capillary membranes	Removal of aqueous phenol, bioremediation of phenolic effluent	Shao et al. (2007) and Edwards et al. (1999)
Lactases	Chitin, chitosan magnetic microspheres	Demineralized whey permeate, hydrolyze milk	Illanes et al. (1990) and Qian et al. (2011)
Tyrosinase	Chitosan clay	Phenol removal	Dinçer et al. (2012)
Lipase	Chitosan	Synthesis of butyl and hexyl oleate	Abd-Elhakeem et al. (2014)
Proteases	Chitosan	Anti-biofilm	Elchinger et al. (2015)
Parathion hydrolase	Chitosan beads	Detoxification of organophosphorus compound	Milani et al. (2015)
Laccase	Chitosan coated magnetic nanoparticles, chitosan	Chlorpyrifos, polyaromatic hydrocarbons, to detoxify industrial effluents, paper and pulp, textile and petrochemical industries, and bioremediation of herbicides, pesticides and certain explosives in soil	Das et al. (2017) and Bautista et al. (2015)
Manganese peroxidase	Chitosan beads	Decolorization of textile effluent	Bilal et al. (2016)
Lignin peroxidase	Chitosan beads	Decolorization of textile effluent	Sofia et al. (2016)
Chloro-peroxidase	Chitosan	Degradation of organic pollutants, degradation and detoxification of an aromatic pollutant	Husain and Ulber (2011) and Alneyadi et al. (2017)

nanoparticles and their potentials as suitable supports for enzyme immobilization. Results indicated that activity of immobilized enzymes and performance of enzyme immobilization onto chitosan nanoparticles are higher than chitosan macro and microparticles. In contrary to other polymeric nanoparticles, chitosan nanoparticles immobilized enzymes strongly increase the stability of immobilized enzymes and their ease of separation from the reaction mixture at the end of the bioprocess completion (Malmiri et al. 2012). Such technologies are relevant to assess effectiveness of an immobilization technique and development of future enzyme immobilization approaches. Lignolytic enzymes immobilized on chitosan coated magnetic nanoparticles was developed to hydrolyse the lignin. Freshly prepared magnetic nanoparticles were coated with chitosan, due to electrostatic attraction between negative charge on freshly prepared magnetic nanoparticles and positive charged on chitosan and nature of chitosan provides amino groups at the surface of functionalized

magnetic nanoparticles and helps in the attachment of molecules like glutaraldehyde spacer or enzyme (Zhu et al. 2008; Kumar 2014).

Thus, it is inferred from the above discussed studies that chitosan immobilized enzyme has strengthened the agri-food industry by providing sustainable development research in agriculture.

4.6 Conclusion

Enzyme immobilization is an area of active research where the recent advances in the design of immobilization support have enabled more precise control of enzyme property. Many enzymes have been successfully immobilized on many different types of organic and inorganic carriers such as alginate beads, polyaniline matrix, glass beads, microporous polypropylene hollow fiber membranes, activated carbon, chitosan film, modified microfiltration membrane and magnetic chitosan microsphere (Domínguez et al. 2007). The selection of the suitable carrier material is another big challenge in immobilization process. Immobilization onto a carrier is a costly affair due to addition of external carrier.

Chitosan is biodegradable in nature and its use is in good conformity with sustainable development. Moreover, it is abundant and its renewable nature make it an attractive and cost-effective biomaterial for the immobilization of many enzymes used in various industries. Moreover, it can replace the some cross-linkers used which are used in cross-linking of enzymes such as glutaraldehyde, due its biocompatibility makes it an environmentally harmless reactant that does not put the health of the workers at risk over glutaraldehyde. Some findings are also indicated that the stability and biodegradation capability of free fungal laccase enhanced by entrapping the enzyme within genipin-activated chitosan beads.

This chapter may be concluded with discussion that enzymes immobilized on chitin- and chitosan-based supports, as compared to unimmobilized biomolecules, has improved considerably the biocatalytic potential that has expanded applications of this versatile biomaterials ranges from biomedical to sustainable agriculture.

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Chapter 5
Chitin and Chitosan Derivative Membranes in Resources, Energy, Environmental and Medical Field

Tadashi Uragami

Abstract In this chapter, the fundamentals of membrane permeation and separation in several membrane technologies are introduced because their understanding is very important for the development of further noble membranes. In dialysis, chitosan derivative membranes for diffusion dialysis and hemodialysis are introduced. In reverse osmosis, a novel ultra low energy reverse osmosis membrane modified by chitosan with glutaraldehyde crosslinking is developed. In nanofiltration, an application of chitosan membranes for the removal of heavy metal ions is investigated. In ultrafiltration, the relationship among the degree of heparinization of chitosan membrane, ultrafiltration characteristics and water content is discussed. In pervaporation, the characteristics of permeation and separation for aqueous ethanol solution through the chitosan and glutaraldehyde crosslinked chitosan membranes in pervaporation is discussed in detail from the viewpoints of chemical and physical structure of membrane. The water-permselective, organic-permselective and organic-organic separation membranes are introduced. In evapomeation, effects of ethanol concentration in the feed vapor on the permeation and separation characteristics and degree of swelling of the chitosan and crosslinked membranes by evapomeation are investigated. In temperature difference–controlled evapomeation, the characteristics of permeation and separation for aqueous dimethyl sulfoxide solution through nonporous and porous chitosan membrane are investigated. Mechanisms for the permeation and separation through these membranes are discussed in detail. In carrier transport, the uphill transport of halogen ions and organic ions such as benzoate ion, amino acids such as phenylalanine, nucleic acid bases such as adenine, cytosine, guanine, uracil is discussed in detail. In catalytic membranes urea hydrolysis by the urease immobilized chitosan membrane is introduced. In fuel cell chemically modified chitosan membranes are introduced.

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Keywords Chitin · Chitosan · Their derivatives · Membrane · Membrane technology · Dialysis · Reverse osmosis · Nanofiltration · Ultrafiltration · Pervaporation · Water-permselective · Organic-permselective and organic-separation membranes · Evapomeation · Temperature difference-controlled evapomeation · Carrier transport · Gas permeation · Fuel cell · Optical resolution

5.1 Introduction

Chitin is the second most important natural polymer in the world. The main sources exploited are two marine crustaceans, crabs and shrimp. Chitosan is one of the few basic polysaccharides that is easily obtained by hydrolyzing chitin.

Chitin and chitosan have high organic solvent resistance, which is advantageous for separation membranes used with organic solvents, where chemical resistance is typical. Specifically, chitin is highly acid resistant and chitosan is highly alkaline resistant. These characteristics make it possible for chitin and chitosan to be used as separation membranes for a variety of uses in response to specific requirements.

In this chapter, the fundamentals and the characteristics of the derivative membranes of chitin and chitosan are described. These derivative membranes are expected to be fundamentally functional material that can support the development of scientific technology in the future.

5.2 Fundamental of Membrane Permeation and Separation

It is very important for the development of novel membranes to understand the fundamental of membrane permeation and separation.

5.2.1 Dialysis

The principle of diffusion dialysis is shown in Fig. 5.1. Diffusion dialysis can transport by simple diffusion according to the concentration gradient of solutes, those with a low molecular weight from a higher concentration solution side to the lower one, but not solutes with high molecular weight. This technique is applied to removal of small molecules in a mixture consisted of macromolecule and small one; for example, removal of sulfonic acid in the synthesis of polystyrene sulfonic acid, and blood dialysis.

5.2.2 Reverse Osmosis

When a solution containing solutes and only one solvent is set up across a semipermeable membrane, an osmotic pressure occurs. When a higher pressure than this osmotic pressure is applied on the side of the solution, only the solvent in the

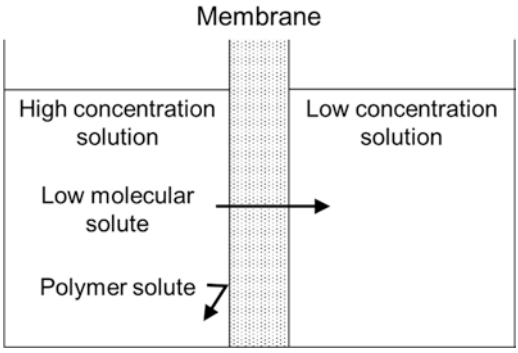


Fig. 5.1 Principle of diffusion dialysis

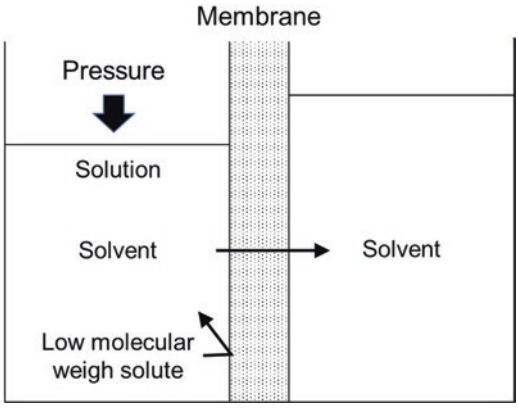


Fig. 5.2 Principle of reverse osmosis

solution can be permeated from the solution side to the solvent through the semipermeable membrane and a solute in the solution can be rejected by the semipermeable membrane, as shown in Fig. 5.2. This membrane-separation technique is called "reverse osmosis" (RO) and is applied for the desalination of seawater and brackish water.

5.2.3 Nanofiltration

The principle of nanofiltration (NF) is the theoretically same as RO, but NF membrane does not almost reject monovalent ion, rejects multivalent ions such as Ca^{2+} and Mg^{2+} and permeate water. Therefore, a higher driving force in the pressure driven separation process is required for RO system but not for NF system. Consequently, the membrane strength required for practical applications makes a great difference.

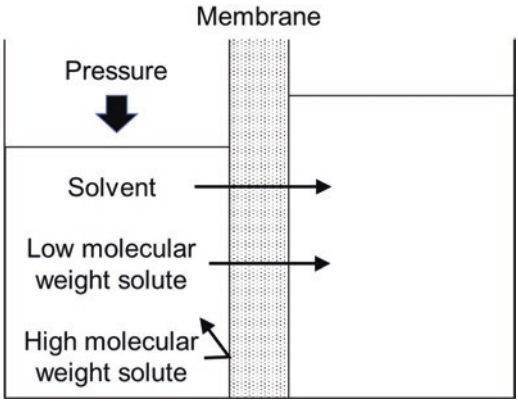


Fig. 5.3 Principle of ultrafiltration

5.2.4 Ultrafiltration

The principle of ultrafiltration (UF) is shown in Fig. 5.3, in which the low molecular weight solutes, such as inorganic salts and organic low molecular weight compounds can be permeated with the solvent through an ultrafiltration membrane, but the high molecular weight solutes such as protein and polysaccharide cannot. In UF, since the separable materials by UF are polymer solutes, the osmotic pressure is much lower than that in reverse osmosis. Consequently, in general, the operating pressure is about 5–50 pa.

5.2.5 Microfiltration

The principle of microfiltration (MF) is shown in Fig. 5.4. Low molecular weight solutes such as inorganic salts, organic low molecular weight compounds and polymer solutes can be permeated with solvent through a MF membrane, but large molecules such as colon bacillus, staphylococcus and AIDS virus cannot pass through the MF membrane. With MF, since separable materials by MF are large molecules, an osmotic pressure is created which is much less powerful than that created in UF. Consequently, in general, the operating pressure is less than 200 kPa. MF usually serves as a pretreatment for other separation processes, such as UF.

5.2.6 Pervaporation

The principle of pervaporation (PV) is shown in Fig. 5.5. A can be seen from this figure when feed mixtures are added on one side of the membrane and the other side is evacuated, a certain component in the feed mixture can be preferentially

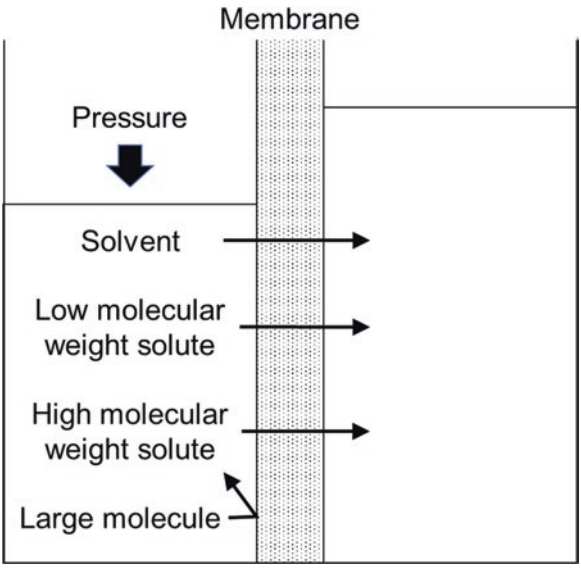


Fig. 5.4 Principle of microfiltration

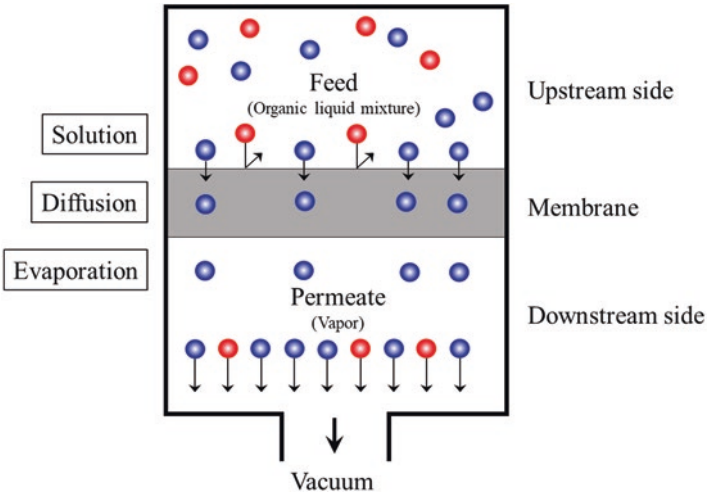


Fig. 5.5 Principle of pervaporation

permeated through the membranes (Binning et al. 1961; Choo 1961). In the pervaporation, the difference in the solubility of permeants into the membrane, the diffusivity of permeants in the membrane, and the relative volatility of permeants from the membrane can influence the characteristics of permeation and separation. This pervaporation technique is advantageous for the separations of azeotropic mixtures, close-boiling point mixtures, and structural isomers.

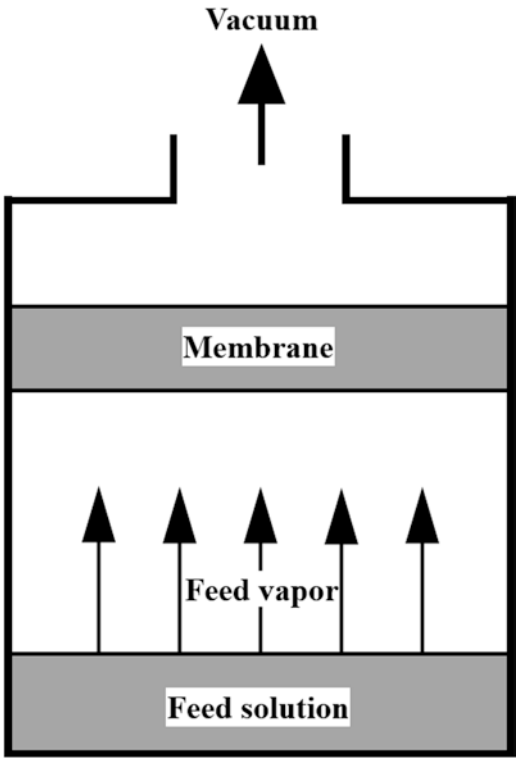


Fig. 5.6 Principle of evapomeation

5.2.7 Evapomeation

Uragami group (Uragami et al. 1988; Uragami and Saito 1989) proposed an "evapomeation" method as a new membrane separation technique, which makes use of the advantage of PV and simultaneously removes a fault of PV as shown in Fig. 5.6. In this evapomeation (EV) technique, the feed solutions are fed without direct contact with the polymer membrane, and only vapor is supplied to the polymer membrane and the swelling or shrinking of polymer membranes due to the feed solutions are prevented.

5.2.8 Temperature-Difference-Controlled Evapomeation

Uragami and Morikawa (1989, 1992) also developed the temperature-difference controlled evapomeation (TDEV) which is controlled the temperatures of the feed solution (I) and the membrane surroundings (II) as shown in Fig. 5.7.

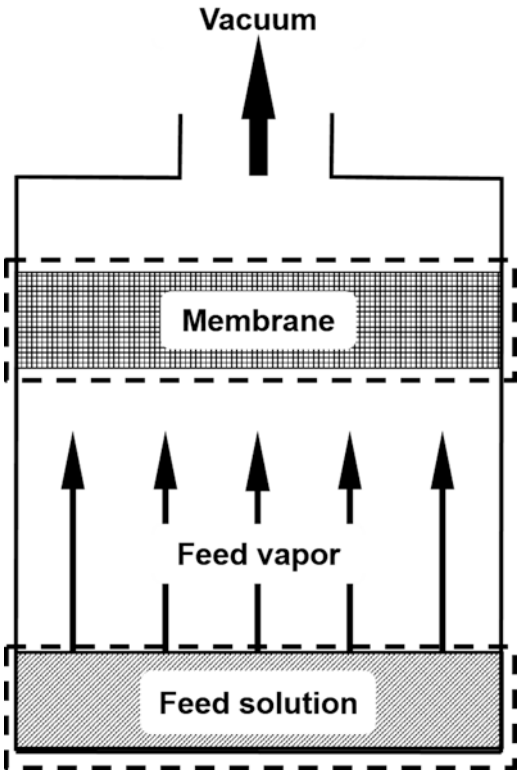


Fig. 5.7 Principle of temperature difference-controlled evapomeation (TDEV)

5.2.9 Carrier Transport

Material transport through membranes is, in general, classified into three fundamental types, as shown in Fig. 5.8 (Uragami 1992).

Figure 5.8a is a model for the passive transport that transfers the material, S, from the left side (L side), with high concentration, to the right side (R side), with low concentration, according to its concentration gradient across the membrane.

In Fig. 5.8b, the carrier, C, in the membrane positively incorporates the material, S, into the membrane by forming a complex, CS, between C and S. In this transport system, in addition to the passive transport in (a), transport with the formation of the complex is added. Consequently, since the transport of material is facilitated, it is called facilitated transport. In such transport, if the carrier can form a complex with a specific material, selectively facilitated transport is possible. In both Fig. 5.8a, b, the material could be transported from the high-concentration side (L side) to the low side (R side) but is never transported because the concentrations are equal on the two sides of the membrane.

A model of an active transport is shown in Fig. 5.8c. In this case the material is actively transported from the low-concentration side (L side) to the high-concentration side (R side) across the membrane against the concentration gradient between both

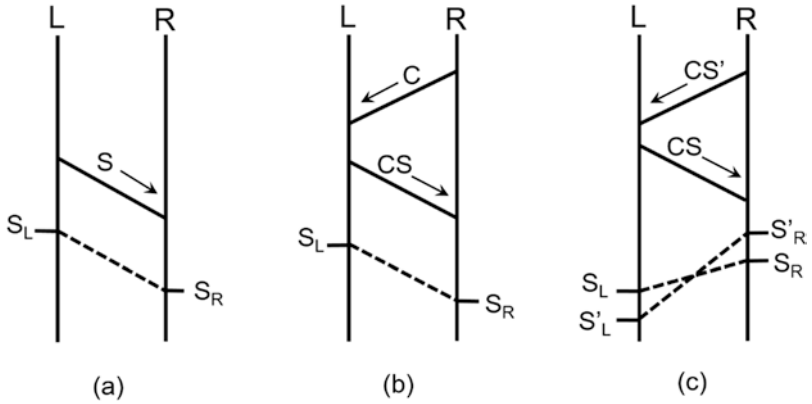


Fig. 5.8 Fundamental types of membrane transport (**a**) Passive transport (**b**) Facilitated transport (**c**) Active transport

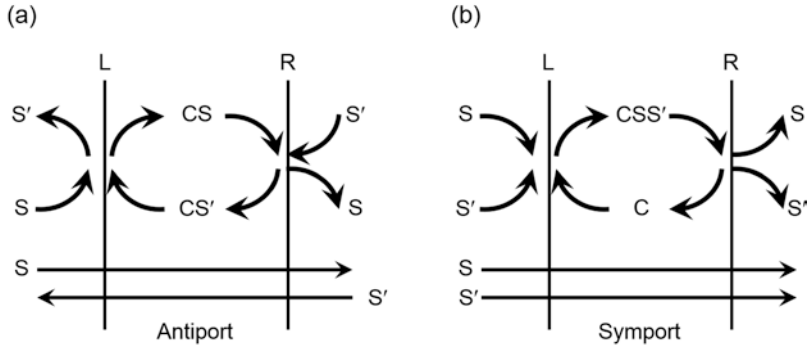


Fig. 5.9 Type of active transport

sides. In this transport form, the material, S, is transported according to the concentration gradient of the complex, CS, in the membrane. This active transport of S is attributed to the conjugated energy for the transport of the complex CS, between the species S′ on the R side and the carrier C from the R side to the L side.

There are two types of active transport, as shown in Fig. 5.9 (Uragami 1992), Fig. 5.9a is an antiport (counter transport) for active transport of the species S and S′. Fig. 5.9b is a symport (cotransport) for active transport of the species S and S′. The active transport of species S in both cases requires conjugated energy due to the transport of species S′.

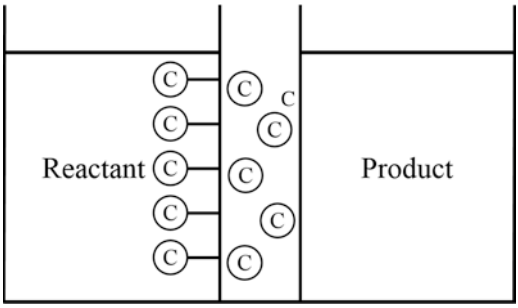


Fig. 5.10 Principle of catalytically functionalized separation through a membrane

5.2.10 Catalytically Functional Membrane

Characteristics of catalytically functionalized membrane systems have both reaction and separation functionalities. As shown in Fig. 5.10, membranes with an immobilized catalyst in the membrane or on the membrane surface can catalyze reactions and the products can be separated through the membrane on the opposite side (Uragami and Aketa 1989). Typical catalytically functionalized separation membranes are the enzyme immobilizing membrane.

5.2.11 Gas Permeation

The driving force in the gas permeation is the difference in gas partial pressures across the membrane, and the gas separation through the membrane is due to the differences in the solubilities of gases into the membrane and in the diffusivities of gases through the membrane, as shown in Fig. 5.11.

5.2.12 Fuel Cell

The fuel cell is similar to ‘a battery’, but it is also ‘an electrical generator’. However, unlike a dry cell battery they are not disposable, and if hydrogen and oxygen are available then electricity can be made continually, as shown in Fig. 5.12.

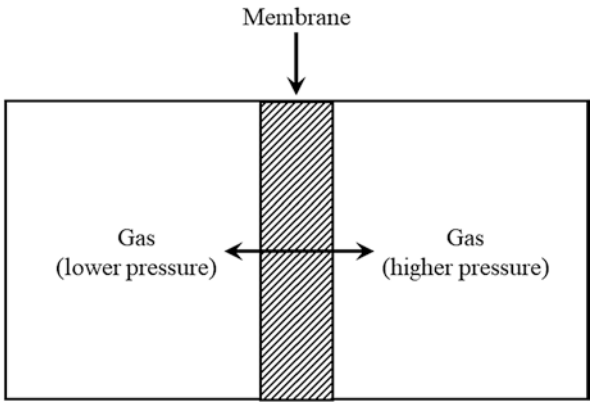


Fig. 5.11 Principle of gas permeation through a membrane

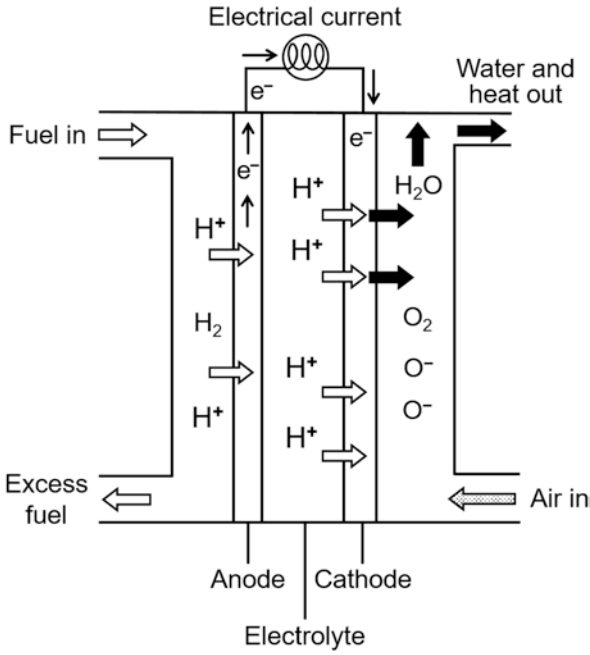


Fig. 5.12 Fundamental type of fuel cell

5.3 Technology

5.3.1 Technology of Dialysis

Chitosan and modified chitosan membranes for ultrafiltration and dialysis were prepared by the casting method with various acids as the salt-forming agents. Sulfamic acid and glutamic acid, as well as some monocarboxylic acids, were found to be excellent salt-forming agents. To improve the solvent-resistant properties of the CS

membranes, two chemical procedures for the modification were employed. One is the cross-linking of the membrane by the photochemical reaction with an aromatic diazido-compound, and the other is chemical modification of CS by carbamoylation. The latter procedure was especially effective; thus a membrane which exhibited high performance and high solvent-resistance could be fabricated (Matsuda et al. 1988).

Chitosan-poly(ethylene oxide) blend membranes, using different molecular weights of poly(ethylene oxide) (PEO), were developed for improved permeability and blood compatibility (Amiji 1995). The equilibrium hydration increased from 44.7% for chitosan to 62.5% for chitosan-PEO blend membranes when the molecular weight of PEO was 10,000 (10 K) or higher. An increase in the hydration of PEO blend membranes was due to intermolecular association between PEO and chitosan chains. Scanning electron microscopy showed that chitosan-PEO membranes were highly porous with size ranging from 50 to 80 nm in diameter observed in membranes made with PEO10K. Electron spectroscopy for chemical analysis suggested an increase in PEO on the membrane surface with increasing molecular weight in the blend. The permeability coefficient of urea increased from 5.47×10^{-5} cm^2/min in chitosan to 9.86×10^{-5} cm^2/min in chitosan-PEO10K membranes. The increase in permeability coefficient could be either due to an increase in the hydrophilicity or the high porosity of the membranes. Although chitosan-PEO membranes did not prevent serum complement activation, platelet adhesion and activation were significantly reduced. Chitosan-PEO blend membranes, therefore, appear to be beneficial in improving the permeability of toxic metabolites and in reducing the thrombogenicity for haemodialysis (Fig. 5.13).

The permeability and diffusion of vitamin B_{12} in chitosan, crosslinked chitosans, and chitosan/poly(vinyl alcohol) blends were studied using "lag time" technique. Apparently the diffusion coefficient, D, for both crosslinked and blended chitosan membranes depends solely upon the equilibrium swelling ratio, Q, of the material in water. The functional dependence of D on Q was obtained from the data. The partition coefficients calculated for vitamin B_{12} in all membranes studied were nearly constant ($K \approx 0.4$). The results are shown to be consistent with the "pore type" transport mechanism for vitamin B_{12} in these chitosan membranes (Nakatsuka and Andrady 1992).

The permeabilities of low molecular weight metabolites were determined through chitosan and a series of chitosan-poly(vinyl pyrrolidone) membranes. The dialysis studies were carried out *in vitro* using a diaphragm type test cell. The basic metabolites (urea, creatinine, and glucose) show higher permeation rates than do the acidic metabolites (uric acid and phosphate) through all the modified membranes. The hydrophilicity of the membranes, molecular weight, and chemical nature of the metabolites were important parameters in determining transport properties of the membranes. It was observed that higher permeation rates can be obtained by manipulating the amount of poly(vinyl pyrrolidone) (PVP) in the blended membranes. The PVP weight loss in the aqueous medium was negligible (Qurashi et al. 1992).

A series of membranes are prepared by air drying thin films, which were composed of poly(vinyl alcohol) (PVA) blended with chitosan [a (1 → 4)2-amino-2-de

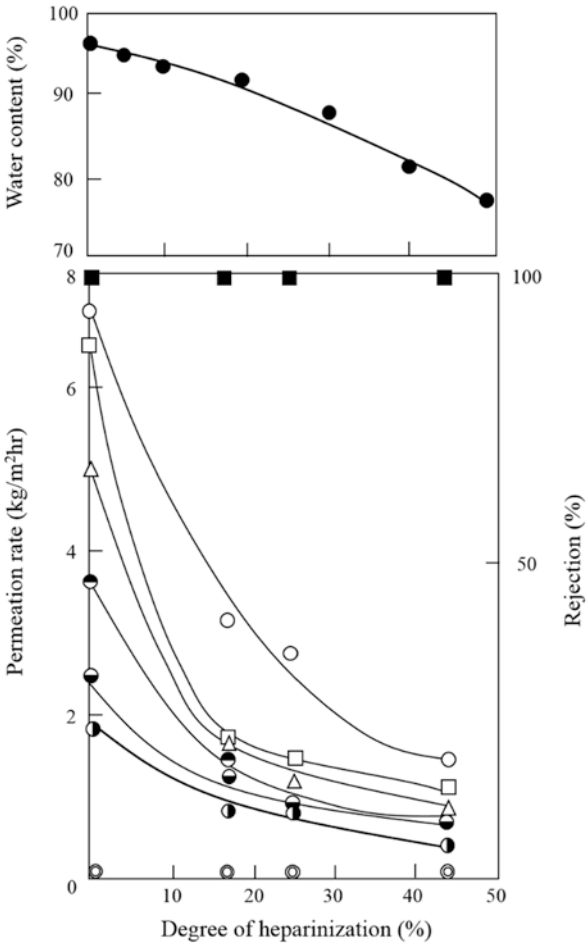


Fig. 5.13 Relationship among the degree of heparinization of chitosan membranes, ultrafiltration characteristics and water contents. ○, □, △, ◓, ◒, ◑ and are the permeation rate for aqueous urea, creatinine, glucose, BSP, vitamin B_{12}, and albumin respectively. ◎ is the rejection for urea, creatinine, glucose BSP and vitamin B_{12}. ■ is the rejection for albumin. ● is the water content. Operating condition are 37 °C, 1 kg/cm^2

oxy-β-D-glucan] (PVA–Chit) in different ratios. The PVA blended chitosan membranes showed improved strength properties and permeability functions for low molecular weight compounds. Nonthrombogenic PVA–Chit (4: 6) membranes were derived by immobilizing bioactive molecules like PGE_1 on heparin-modified membranes, via free radical mechanisms, by N_2 plasma. This novel membrane demonstrated good permeability properties for small molecules and showed a dramatic reduction in platelet attachment. The prostaglandin E_1 immobilized substrate also indicated an increase in albumin surface attachment and a reduction in fibrinogen binding. This may be one of the parameters for a reduced platelet surface attachment, which may also improve the blood compatibility of the substrate. It is also postulated that the total water content of membranes need not be the prime factor governing the permeability of solutes through water-swollen membranes. However, many other parameters govern the solute permeability, like the amount of solutes dissolved in bound water and the status of water in the polymer matrix (Chandy and

Sharma 1992). The diffusion coefficient for both the cross-linked and blended chitosan membranes was solely dependent on the equilibrium swelling ratio of the membrane in water. The transport mechanism for vitamin B_{12} in these chitosan membranes was consistent with the "pore type." The equilibrium sorption of various sodium salts for chitosan, *N*-benzoylchitosan, and *N*-octanoylchitosan membranes was measured and explained by a dual mechanism, consisting of partition and Langmuir sorption (Seo et al. 1995).

Chitosan-poly(ethylene oxide) (PEO) blend membranes, using different molecular weights of PEO, were developed for improved permeability and blood compatibility. The equilibrium hydration increased from 44.7% for chitosan to 62.5% for chitosan-PEO blend membranes when the molecular weight of PEO was 10,000 (10 K) or higher. An increase in the hydration of PEO blend membranes was due to intermolecular association between PEO and chitosan chains. Scanning electron microscopy showed that chitosan-PEO membranes were highly porous with size ranging from 50 to 80 nm in diameter observed in membranes made with PEO10K. Electron spectroscopy for chemical analysis suggested an increase in PEO on the membrane surface with increasing molecular weight in the blend. The permeability coefficient of urea increased from 5.47×10^{-5} cm^2/min in chitosan to 9.86×10^{-5} cm^2/min in chitosan-PEO10K membranes. The increase in permeability coefficient could be either due to an increase in the hydrophilicity or the high porosity of the membranes. Although chitosan-PEO membranes did not prevent serum complement activation, platelet adhesion and activation were significantly reduced. Chitosan-PEO blend membranes, therefore, appear to be beneficial in improving the permeability of toxic metabolites and in reducing the thrombogenicity for haemodialysis (Amiji 1995).

The effects of the kinds of acids used to dissolve chitosan, the chitosan concentration, the drying time of the dope solution, and the kinds of gelating agents acting on the membrane structure $_A$ and their performance were studied in detail. On increasing the chitosan concentration, the solute permeability decreased while the selectivity of theophylline to vitamin B_{12} increased. The membrane changed from the wholly porous structure to the asymmetric structure by an increase of the chitosan concentration. Furthermore, the use of ethanol as the gelating agent brought about a wholly porous structure with a high permeability and a low selectivity. The asymmetric structure and the wholly dense structure were obtained in the cases of the gelating agents, such as the aqueous NaOH solution and dimethyl sulfoxide, respectively (Matsuyama et al. 1999a). The permeabilities of three kinds of solutes of similar sizes, such as anionic benzenesulfonic acid, neutral styrene glycol, and cationic theophylline in the chitosan membrane were investigated. Benzenesulfonic acid showed the highest permeability, whereas theophylline showed the lowest, although these solutes have almost the same size. This could be explained by the electrostatic attraction or repulsion between the solute and the membrane instead of the size-exclusion effect. The permeabilities of benzenesulfonic acid and theophylline increased and decreased, respectively, with the decrease of pH from 7.4 to 4.0 because of the increase of the charge density of the membrane. Thus, the selectivity of benzenesulfonic acid to theophylline increased and reached about 30 at pH 4.0 (Matsuyama et al. 1999b).

The effects of membrane composition and the external pH on the swelling and the drug permeation behavior of interpenetrating polymer network (IPN) membrane could be summarized as follows; chitosan incorporated into tetraethoxysilane (TEOS)-IPN swelled at pH 2.5 while shrunk at pH 7.5. This swelling behavior was completely reversible and the membrane responded rapidly to the change in environmental pH condition. According to swelling behavior, an increase in pH from 2.5 to 7.5 yielded an increase in the rate of drug permeation because of the shrinking of the incorporated chitosan in TEOS-IPN in low permeation rate. The optimal TEOS-chitosan ratio for maximum pH-sensitivity existed and drug permeation was influenced not only with the external pH but also with the ionic interactions between the drug and membrane (Park et al. 2001).

Blended membranes of chitosan and PVA in various ratios were prepared and treated with formaldehyde. Electron spectroscopy for chemical analysis of the membrane showed that the NH_2 group in chitosan changed into N C group after membrane treatment with formaldehyde. From the spectral change of Fourier-Transform Infrared Spectroscopy (FTIR), the hydroxyl groups disappeared and an acetal ring and ether linkage were formed for the reaction between the hydroxyl groups of PVA and formaldehyde. The results of differential scanning calorimetry (DSC), dynamic mechanical analysis (DMA) and thermogravimetric analysis (TGA) showed that (1) the crystalline area in PVA decreased after treatment with formaldehyde, (2) chitosan and PVA are not very compatible in the chitosan/PVA blended hydrogel membrane and (3) the thermostability of the membranes is enhanced by formaldehyde as crosslink agent. The effect of chitosan content on the water content and water vapor transmission rates on the blended hydrogel membrane were determined. It was found that the water content and water vapor transmission rates on the blended hydrogel membrane increased with increasing chitosan content. In antibacterial assessment, it appears that the antibacterial activity of all chitosan/PVA blended hydrogel membranes is similar. The viable cell number of aurococcus on the various chitosan/PVA blended hydrogel membranes was about $(2.5 \pm 0.5) \times 10^7$ cells/ml. The permeation of creatinine, 5-FU, uric acid and vitamin B_{12} through the chitosan/PVA blended hydrogel membranes were conducted. The linear relationship between permeability of creatinine, 5-FU and vitamin B_{12} molecules and chitosan content in the chitosan/PVA blended hydrogel membranes were found. As the electrostatic attraction between the uric acid and chitosan in the membrane, the permeation of uric acid through the chitosan/PVA blended hydrogel membranes is higher for the membranes with chitosan content higher than 80% in the blended hydrogel membranes.

Blend membranes of chitosan and PEO with different molecular weights of 100,000 and 600,000 were prepared by the solution cast technique (Nasir et al. 2005). The chitosan-PEO blend membranes were produced to study their water adsorptions capacity and characteristics of the haemodialysis membrane application. An increase in the water adsorption capacity of chitosan-PEO blend membranes compared to the pure chitosan was due to the porous structure as evident from the scanning electron micrograph. Addition of PEO with higher molecular weight had reduced the percentage of water adsorption of the chitosan-PEO

blend membranes. X-ray powder diffraction (XRD) results revealed that chitosan-PEO blend membrane with higher water adsorption ability shows lesser degree of amorphosity. Intermolecular interactions between chitosan and higher molecular PEO chains in the blend contributed to important alteration in chitosan structure as observed in the infrared spectroscopy which lessens the permeability of the membrane.

Chitosan was graft copolymerized with 2-hydroxyethyl methacrylate (HEMA) for the development of blood-compatible dialysis membranes (Radhakumary et al. 2006). The permeation characteristics of HEMA-grafted CS films for four different solutes creatinine, urea, glucose, and albumin was studied in vitro at 37 °C for the assessment of the suitability as dialysis membranes. The grafted film CH-12.5 composition (425% grafting) showed very high permeation to creatinine by reaching the equilibrium within 45 min. The compositions CH-7.5 and CH-12.5 showed excellent permeation to glucose when compared to virgin CS films. In the case of urea permeation, all the grafted compositions exhibited higher percent permeation than the virgin CS films. The copolymer films CH-7.5 and CH-12.5 showed enhanced permeability for the high-molecular weight solute, albumin. The other grafted copolymer compositions followed almost the same trend as that of CS for the low-molecular weight solutes as well as the high-molecular weight solute. The copolymer films were also found to be highly blood compatible, noncytotoxic, and biodegradable. Hence, the need for developing blood- compatible CS membranes with desirable permeability properties is achieved by the graft copolymerization of HEMA onto CS.

Chitosan was graft copolymerized with vinyl acetate using ceric ammonium nitrate as the initiator. The chitosan-*g*-poly(vinyl acetate) (chitosan-*g*-PVAc) membranes were found to be blood compatible, noncytotoxic, and biodegradable. The physicochemical characterization of the membranes revealed that the membranes possess the synergistic effect of the natural synthetic hybrids of chitosan and PVAc with excellent mechanical stability and tunable hydrophilic/hydrophobic characteristics. The permeation characteristics of chitosan-*g*-PVAc membranes for four different solutes creatinine, urea, glucose, and albumin was studied *in vitro* at 37 °C for assessment of the suitability of them as hemodialysis membranes. The studies showed that the membranes exhibit higher permeability to creatinine, urea, and glucose compared with the commercial cellulose membrane and are impermeable to the essential nutrient albumin. Hence, the need for the development of biocompatible, mechanically strong dialysis membranes could be addressed with the modification of chitosan through grafting with PVAc. Potential applications like artificial kidney, artificial pancreas, and so forth, are envisaged from these membranes (Radhakumary et al. 2011).

The effect of membrane dialysis on the characteristics of chitosan based lyophilised wafers was investigated. Gels loaded with bovine serum albumin (BSA), glycerol and D-mannitol were lyophilised with or without membrane dialysis and characterised by X-ray diffraction, attenuated total reflectance FTIR spectroscopy, circular dichroism, scanning electron microscopy (SEM) hydration capacity, *in vitro* mucoadhesivity and drug dissolution. The dialysed wafers demonstrated

enhanced mucoadhesion and drug release properties while newly formed sodium acetate in the undialysed wafers caused increased crystallinity with poor mucoadhesion and drug release properties. Removal of sodium acetate by membrane dialysis is essential for obtaining optimised wafers for potential application to the buccal mucosa surface (Ayensu et al. 2012).

A series of membranes are prepared by solvent drying method, which were composed of *N*-carboxymehtyl chitosan blended with poly(vinyl alcohol) [N-CMC/PVA]. The *N*-CMC/PVA membranes showed improved strength properties and permeability for low-molecular weight compounds. These novel membranes exhibited good permeability properties for small molecules also indicated a decrease in protein adsorption on the surface of membrane. The structure and the morphology of the resulting membranes were characterized by FTIR, NMR, elementary analysis and SEM (Lusiana et al. 2013).

Alkane (petroleum ether) vapor plasma technique was used for surface modification of chitosan membranes to control their permeation rate of water-soluble drugs and metabolites. Water contact angles of the chitosan surface increase from 13° to 23° after plasma treatment at 93 W for 60 min, and from 13° to 26° after plasma treatment at 119 W for 30 min, indicating reduced hydrophilicity of the membrane surface. Mechanical properties such as tensile strength and elongation-at-break of the chitosan membranes were also improved. In particular, there was a 6–7 fold increase in tensile strength in the wet state for the chitosan membrane treated at 93 W for 30 min. Permeation coefficients through the chitosan membrane plasma treated at 93 W for 30 min for urea, creatinine, uric acid, and *cis*-DDP decreased by 54.0%, 83.3%, 64.7% and 47.6%, respectively (Wang et al. 2001).

Chitosan (CS)/gelatin (Gel)/polyvinyl alcohol (PVA) hydrogels were prepared by the gamma irradiation method for usage in wound dressing applications. Chitosan and gelatin solution was mixed with PVA solution at different weight ratios of CS/Gel of 1:3, 1:2, 1:1, 2:1 and 3:1. The hydrogels irradiated at 40 kGy. The structure of the hydrogels was characterized by using FT-IR and SEM. The CS/Gel/PVA hydrogels were characterized for physical properties and blood clotting activity. The tensile strength of CS/Gel/PVA hydrogel enhanced than on the basis of the Gel/PVA hydrogel. The highest tensile strength reached the 2.2 Mpa. All hydrogels have shown a good coagulation effect. It takes only 5 min for the BCI index to reached 0.032 only 5 min when the weight ratio of CS/Gel was 1:1. It means that the hemostatic effect of hydrogels were optimal. And the hydrogels also showed good pH-sensitivity, swelling ability and water evaporation rate. Therefore, this hydrogel showed a promising potential to be applied as wound dressing .

The composite membrane was produced by mixing the chitosan and sodium alginate solutions followed glutaraldehyde as a crosslinking agent for filtration of Ca^{2+} ion, phosphate ion, sodium salicylate, urea, and albumin. The membranes were characterized by FTIR to verify the functional group, tensile testing to test their mechanical stability, the surface morphology analysis, and application of membrane for filtration that minerals. The results showed that the mechanical properties of the

membrane the higher the concentration of chitosan in the membrane, the higher the value of modulus young. The morphology analysis showed that this membrane has a diameter of the pore in range nanopore, included in the range of ultrafiltration membrane. The membrane of chitosan–glutaraldehyde alginate was applied for the filtration some minerals with a variety of concentrations. This membrane could filtration Ca^{2+} and phosphate ion, sodium salicylate, urea and albumins were 5.51 mmol/L, 39.27 mg/L, 30.87 mg/L, 500.56 mg/L and 0 mg/L, respectively.

The membranes of *O*-(carboxymethyl)-chitosan (*O*-CMC), *O*-CMC-graphene oxide (GO) and *O*-CMC-reduced graphene oxide (rGO) are prepared by dialysis method, respectively. The membrane structures and properties are investigated with different techniques. FT-IR and XRD spectra indicated that the composition (*O*-CMC) of the membranes is protonated. The mechanical strength of these membranes is enhanced with the added amount of GO. These membranes show effective adsorption capacity for dyes and Cu^{2+} in water, showing that they may be the promising applications for water purification including dye adsorption and selective dialysis (Huang et al. 2018).

5.3.2 Technology of Reverse Osmosis

Alkali resistant reverse osmosis membranes were fabricated by spreading solutions of chitosan, a poly N-acetyl glucosamine, in 2.0% acetic acid on a glass plate. The membrane had a flux rate of 1.67 ± 10^{-3} $cm^3/cm^2/sec$ and a salt rejection capability of 78.8% with 0.2% NaCl at 680 psi. Addition of 40% polyethylene glycol to the membrane casting solution increased permeability, while 10% chloromethyl oxirane improved durability of the membranes (Yang and Zall 1984).

N-phthaloylchitosan was synthesized by the reaction of chitosanX.35 with phthalic anhydride in dimethyl formamide. Different compositions of polysulfone (PSf) and *N*-phthaloylchitosan were used to prepare novel polysulfone/*N*-phthaloylchitosan composite membranes by phase inversion method. The composition ratios between the former and the latter were 80 : 20, 85 : 15, 90 : 10, and 95 : 5. Water flux results revealed that, PSf : *N*-phthaloylchitosan 80 : 20 membrane is found to have greatest effective pore area while PSf : *N*-phthaloylchitosan 95 : 05 membrane has the smallest value. The pore area is found to be larger with the increase in *N*-phthaloylchitosan composition. In addition, its water swelling property increases with the increase of *N*-phthaloylchitosan composition. Water flux results are in consistent with dielectric constant value. Use of known molecular weight of polyethylene glycol rejection study, revealed that, PSf : *N*-phthaloylchitosan 95 : 05 membrane possessed the smallest pore size among these membranes. In conclusion, change of ratio between PSf and *N*-phthaloylchitosan, considerably affects membrane pore size and hydrophilicity. For salt filtration, membrane PSf :

N-phthaloylchitosan 95 : 05 showed 93%, 76.11% and 70.12% rejection of $MgSO_4$, Na_2SO_4, and NaCl, respectively.

EL-Gendi et al. reported with "developing novel polyamide-6/chitosan membranes for water desalting using wet phase inversion technique", were prepared using an appropriate polymer concerning the national circumferences, along with the definition of different controlling parameters of the preparing processes and their effects on the characteristics of the produced membranes. Further, evaluation process of the fabricated sheets was undertaken. Preparation process was followed by assessment of the membrane structural characteristics; then the desalting performance of each prepared membrane was evaluated under different operating conditions in order to find the structure–property relationship. The results show that the membrane flux increases with the increase of operating pressure. The salt rejection and permeation flux have been enhanced indicating that the chitosan addition to the polyamide-6 (PA-6) membrane increases the membrane hydrophilic property. Hydraulic permeability coefficient is not stable and varies considerably with the operating pressure.

Membrane-based desalination is a proven and established technology for mitigating increasing water demand. The high-flux membrane will require lower pressure to produce the given quantity of water and therefore will consume less energy. Raval et al. demonstrated a novel method to produce a high-flux membrane by surface modification of thin-film composite reverse osmosis (TFC RO) membrane. TFC RO membrane was exposed to a sodium hypochlorite solution of 1250 mg/l for 30 minutes and 60 minutes at pH 11.0, followed by 1000 mg/l chitosan for 60 minutes at pH 2.5, and the solute rejection/flux were monitored. It was observed that there is up to 2.5 times increment in flux with *ca.* 3% increase in solute rejection in the case of chitosan-treated membrane. Although the flux increase is more in membrane with longer exposure to sodium hypochlorite, the decline in solute rejection was also significant. The membrane samples were characterized by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) to understand the chemical structural changes in the membrane, atomic force microscopy to understand the morphological changes on membrane surface, zeta potential for surface charge and contact angle analysis to understand the change in hydrophilicity. The % rise in trans-membrane flux per °C rise in feed water temperature was more in the case of chitosan-modified membrane as compared to virgin TFC RO membrane. The higher temperature sensitivity makes it a good candidate for solar powered reverse osmosis, where low grade thermal energy can be utilized to increase feed water temperature, and higher temperature feed water gives more a pronounced advantage in trans-membrane flux.

With excellent mass transport properties, graphene oxide (GO)-based lamellar membranes are believed to have great potential in water desalination. In order to quantify whether GO-based membranes are indeed suitable for reverse osmosis (RO) desalination, three sub-micrometer thick GO-based lamellar membranes: GO-only, reduced GO (RGO)/titania (TO) nanosheets and RGO/TO/chitosan (CTS) are prepared, and their RO desalination performances are evaluated in a home-made RO test apparatus. The photoreduction of GO by TO improves the salt

rejection, which increases slowly with the membrane thickness. The RGO/TO/CTS hybrid membranes exhibit higher rejection rates of only about 30% (greater than threefold improvement compared with a GO-only membrane) which is still inferior compared to other commercial RO membranes. The low rejection rates mainly arise from the pressure-induced weakening of the ion–GO interlayer interactions. Despite the advantages of simple, low-cost preparation, high permeability and selectivity of GO-based lamellar membranes, as the current desalination performances are not high enough to afford practical application, there still remains a great challenge to realize high performance separation membranes for water desalination applications.

Mineral scaling persists in many water treatment processes. More specifically, it can significantly reduce the efficacy of aromatic polyamide (PA) membranes during RO water treatment. Previous studies have integrated hydrophilic materials, such as polyethylene glycol (PEG), onto RO membranes to combat scaling from generally hydrophobic feed water constituents; however, there are still outstanding knowledge gaps regarding the interplay of the modified membrane surface chemistry and the water chemistry in complex RO feed waters. The mechanisms of hydrophilic PEG-grafted PA membranes in reducing mineral scaling from calcium carbonate ($CaCO_3$) and calcium sulfate ($CaSO_4$) in the presence of humic acid (HA) were investigated by Ray et al. Based on surface and solution analyses, the authors found that colloidal formation was significantly reduced on PA–PEG surfaces in systems without HA. When HA was introduced, $CaCO_3$ scaling was reduced on both virgin and PA–PEG membrane surfaces; while, interestingly, synergistic PEG–HA–$CaSO_4$ interactions increased $CaSO_4$ colloidal formation on PA–PEG membranes. Promoted $CaSO_4$ formation results from a high negative surface charge near the PEG-modified membrane surface when HA and SO_4^{2-} are present, attracting more Ca^{2+} to form $CaSO_4$. The results of this work provide new information about colloidal formation at water–membrane interfaces for designing better PEG and PEG-based scale-resistant desalination membranes.

Novel thin film poly(vinyl alcohol)/chitosan (PVA/CS) based reverse osmosis membranes infused with silane crosslinked tetraethylorthosilicate (TEOS) were prepared by dissolution casting methodology. The performance characteristics and the scope of the reverse osmosis membranes were explicated by FTIR, TGA, DSC, SEM, contact angle, XRD and RO permeation tests which determined the functional groups and network of covalent crosslinks, thermal properties, morphology, hydrophilicity, structural investigation and RO properties, respectively. It was found that the membrane surface became smoother, more hydrophilic, with improved thermal stability, increased salt rejection and good permeation flux after the appropriate infusion of TEOS. The crosslinked membranes showed more hydrophilicity compared to the uncrosslinked PVCS membrane. The SEM micrographs of membranes revealed dense structure with no mottled surfaces. PVCS-4 showed an optimal flux of 1.84 L/(m^2 h) and 80% salt rejection that confirmed the selective interaction of TEOS molecules with PVA/CS polymer backbone compared to the pristine (PVCS) membrane. The antibacterial properties of the membranes successfully showed the inhibition of the growth of *Escherichia coli*.

The energy consumption of RO has declined significantly since inception and to further decrease the energy consumption is a challenging task. The present article demonstrates the novel method to increase the membrane productivity and reduce energy consumption of desalination. Thin film composite RO (TFC RO) membrane was subjected to 2000 mg/L sodium hypochlorite for 1 h followed by varying concentrations of chitosan and glutaraldehyde for 1 h each to make a hydrophilic supra-molecular assembly of linear polysaccharide over the polyamide layer. RO membrane exposed to 1000 mg/L chitosan and glutaraldehyde each reported 180% increase in water-flux with about 2.7% increase in divalent ion rejection as compared to virgin TFC RO membrane. The superior performance of the membrane was explained by increased hydrophilicity as shown by decline in contact angle from 46.37° to 29.87°, increase in surface area ratio from atomic force microscope image analysis, and modification in chemical structure of polyamide from ATR-FTIR spectroscopy. It was further investigated that curing of glutaraldehyde treated membrane resulted in decreased water-flux because of increase in crosslink density. Thus, an ultra low energy RO process can be developed based on polyamide–chitosan–glutaraldehyde membrane.

5.3.3 Technology of Nanofiltration

The acetylated chitosan membrane (degree of acetylation: 72%) showed a high rejection and a high permeation rate in nanofiltration for an aqueous solution of 0.2% $CaCl_2$, and had a high chemical reagent, an organic solvent, and heat stability.

Kamiński and Modrzejewska present an application of chitosan membranes for the removal of heavy metal ions. Investigations covered membranes produced by phase inversion. Additionally, separation properties of acetylated membranes were tested. Low-viscous chitosan produced by the Sea Fisheries Institute—Poland used in the experiments. The investigations were carried out for the transition metal ions Cr(VI), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), and Cd(II). A method for metal ions separation by means of chitosan membranes was proposed. The metal ions were complexed in the membrane during ultrafiltration of the solution. The separation ability of the membranes was investigated for individual metal ions and for a mixture. The effect of the pH of the solution on separation properties of membranes was determined. The concentration of metal ions was investigated by the method of inductively coupled plasma (ICP) atomic emission spectrometry. The investigations show the suitability of chitosan membranes produced by the phase inversion method for the removal of metal ions.

Since chitosan molecules have amino and hydroxyl groups, and as the chitosan membrane was cross-linked with polyfunctional compounds such as dicarboxylic acid, diisocyanate, diglycigyl, and cyanul chloride, these cross-linked chitosan membranes were very stable for alkaline and acid solutions and were reverse osmosis membranes with a high water permeability and a high salt rejec-

tion. The resistance of novel surface cross-linked chitosan/poly(acrylonitrile) composite nanofiltration membranes to pH and organic solvents was studied with respect to the effects of cross-linking parameters, namely, glutaraldehyde concentration and cross-linking time. Pure water flux and polyethylene glycol transmission data indicated that at pH 2.5and 11, the membrane stability increased with increasing glutaraldehyde concentration and was much better at pH 11 than at pH 2.5. All surface cross-linked membranes showed a reduced swelling between pH 4–10.

Effects of surface crosslinking of chitosan/poly(acrylonitrile) (PAN) composite nanofiltration membranes at different crosslinker (glutaraldehyde) concentrations and crosslinking times on their surface chemical composition and sieving properties such as pure water permeation, molecular weight cut-off and the rejection of mono/divalent salts and mono/oligosaccharides were investigated. Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR) and X-ray photoelectron spectroscopy (XPS) studies revealed the crosslinking of chitosan with glutaraldehyde as well as variations in chemical composition with glutaraldehyde concentration and crosslinking time. Pure water permeation/swelling in water decreased and rejection of salts and sugars increased with increasing glutaraldehyde concentration, indicating pore contraction and increase in hydrophobicity as well as pore tortuosity due to crosslinking. Molecular weight cut-offs of surface crosslinked membranes were in the range of 550–700 Da, a characteristic of nanofiltration membranes, whereas uncrosslinked membrane had cut-off of >1500 Da. The crosslinked membranes were found to be stable over 10-h operation for pure water permeation and the stability increased with increasing glutaraldehyde concentration.

Kamiński and Modrzejewska present an application of chitosan membranes for the removal of heavy metal ions. Investigations covered membranes produced by phase inversion. Additionally, separation properties of acetylated membranes were tested. Low-viscous chitosan produced by the Sea Fisheries Institute-Poland was used in the experiments. The investigations were carried out for the transition metal ions Cr (VI), Mn (II), Fe (III), Co (II), Ni (II), Cu (II), Zn (II), and Cd (II). A method for metal ions separation by means of chitosan membranes was proposed. The metal ions were complexed in the membrane during ultrafiltration of the solution. The separation ability of the membranes was investigated for individual metal ions and for a mixture. The effect of the pH of the solution on separation properties of membranes was determined. The concentration of metal ions was investigated by ICP atomic emission spectrometry. The investigations show the suitability of chitosan membranes produced by the phase inversion method for the removal of metal ions.

Chitosan was modified with a chiral compound and a positively charged compound. Series of novel composite NF membranes were prepared by over-coating the polysulfone ultrafiltration membrane with the mixture of chitosan and chitosan derivative (Mu et al. 2006).

The chiral compound, the positively charged compound and their chitosan derivatives were characterised by IRFT and polarimeter. The structure of the membrane

was characterized by SEM. The performance of membrane was strictly related to the chiral compound and the positively charged compound grafted to chitosan and its composition. The rejection of P_{2-7} composite NF membrane reached the maximum of 95·2%, and the flux remained to be as high as 687·4 L/(m^2 h) at 0·4 MPa with 1000 mg/L $CaCl_2$. Electrostatic effect had no effect on NaCl, while excellent effect on $CaCl_2$. It was typical positively charged NF membrane, which was suitable for separating the multivalent cation solutes from the feed solution.

O-carboxymethyl chitosan (NOCC) composite (NF) membranes were prepared by coating and cross-linking. The fermentation effluent from a wine factory was treated with the resulting NOCC/polysulfone (PSF) composite NF membranes. The permeate flux and the removal efficiencies of the resulting NF membranes for the color, chemical oxygen demand (COD_{Cr}), total organic carbon (TOC), and conductivity of the fermentation effluent were investigated in relation to the driving pressure, the feed flow, and the operation time. The permeate flux and the removal efficiencies were found to increase with the increase of the driving pressure or the feed flow. At 0.40 MPa and ambient temperature the removal efficiencies were 95.5%, 70.7%, 72.6%, and 31.6% for color, COD_{Cr}, TOC, and conductivity, respectively. The membrane was found to be stable over a 10-h operation for the fermentation effluent treatment.

NF is considered to be an intermediate process between UF and RO. Chitosan derivative containing carboxylic acid group (-COOH), i.e. *N,O*-carboxymethyl chitosan (NOCC), is prepared by Boricha et al. (Boricha and Murthy 2008). Then, the NOCC composite NF membrane having a polyether sulfone (PES) UF membrane as the substrate is prepared using a method of coating and crosslinking, in which glutaraldehyde solution is used as the crosslinking agent. The developed membranes are characterized in terms of FTIR/ATR, scanning electron microscopy/energy dispersive X-ray analysis (SEM/EDXA), EA, swelling behavior, tensile strength, and thermogravimetric analysis (TGA), which confirm the formation of the target membranes. Rejection efficiency of nickel ions from aqueous solutions, of nickel sulfate and nickel chloride, is investigated. The experiments are carried out for different feed concentrations, feed flow rates and applied pressures and the corresponding permeate flux and rejections are measured. The maximum observed rejection is found to be 80 and 62% of 5 ppm, 78 and 59% of 10 ppm; and 74 and 57% of 50 ppm feed concentration of nickel sulfate water and nickel chloride water systems, respectively. The observed order of the separation of the nickel salts is $NiSO_4$ and $NiCl_2$.

Conventional NF membranes had a relatively low flux (Mu et al. 2012). In this work, two mesogenic compounds were grafted to chitosan in order to change the structure, hence the performance of the NF membrane. A series of novel composite NF membranes were prepared by over-coating the polysulfone ultrafiltration membrane with the mixture of chitosan and mesogenic compounds modified chitosan. The two mesogenic compounds and their chitosan derivatives were characterized by FTIR, DSC, polarized optical microscope (POM); the structure of the membrane was characterized by SEM. The composite NF membrane's rejection rate and flux were strictly related to the mesogenic compound grafted to chitosan and its composition.

Extremely high flux, 2543.3 l/(m^2 h) was observed with P_{2-4} composite NF membrane, and the rejection remained to be as high as 66.3% at 0.4 MPa with 1000 mg/L NaCl. These results, together with SEM and infrared images of the composite NF membrane, indicated that the mesogenic compound structure was crucial for the structure and function of the composite membrane (Mu et al. 2012).

Four nanofiltration membranes, viz., (1) coating of *N,O*-carboxymethyl chitosan (NOCC) on polyethersulfone ultrafiltration (PES UF) substrate membrane; (2) chitosan and acrylonitrile butadiene styrene (ABS) in the blend ratio of 0:100 (ABS); (3) diethylenetriamine pentaacetic acid coating via casting method on PES UF substrate membrane (DC50); and (4) NOCC and cellulose acetate (CA) polymer blend solution (0.4 wt%) coated on a glass plate (NOCC–CA) were used for the separation of mercury and chromium ions. From the experimental data, it is evident that ABS membrane gave highest observed solute rejection (92.88 and 88.67% for 10 ppm feed concentration of mercury sulphate–water system and chromium sulphate–water system, respectively) and NOCC–CA membrane gave highest permeate volume flux. But from the rejection as well as permeate volume flux point of view, NOCC–PES membrane is considered to be the best choice among all the membranes (Mungray and Murthy 2012).

Polysulfone and chitosan blend membranes were prepared by incorporating titanium dioxide nanotubes (TiO_2NT) in different compositions. The proper blending of polysulfone and chitosan in the membranes was confirmed by ATR-IR spectroscopy. The influence of nanotubes on morphology of membranes was investigated by Field Emission Scanning Electron Microscopy (FESEM). The effect of nanotubes on hydrophilicity of the membranes was studied by water swelling and contact angle measurements. The distribution of TiO_2NT on the membrane surface was determined by Transmission Electron Microscope (TEM) analysis. The permeation property of PSf/CS/TiO_2NT membranes was carried out by measuring the time dependent pure water flux (PWF). Bovine serum albumin (BSA) protein rejection studies were performed to know the antifouling properties. The rheological percolation threshold of PSf/CS/TiO_2NT solutions was measured by viscosity studies. The nanotubes incorporated PSf/CS membranes showed enhanced permeation and antifouling properties compared to PSf/CS and nascent PSf ultrafiltration membranes. Membranes prepared well above rheological percolation threshold showed drastic reduction in pore size and acted as NF membranes.

Sulfated chitosan composite NF membranes having PAN UF membrane as the base material are prepared by coating and cross-linking, in which epichlorohydrin (ECH) aqueous solution is used as the cross-linking agent. Newly prepared membranes are characterized by FTIR-ATR, XRD, NMR, TGA, and SEM. Effects of various factors like concentration of cross-linking agent, casting solutions, and membrane preparation conditions on the performance of newly prepared composite NF membranes are studied results showed that the NF membrane with excellent rejection performance has 1.0 wt % of sulfated chitosan concentration, 0.4 wt % ECH concentrations, 1 h cross-linking at 60 °C. With this composite NF membrane, the solutes rejection from electrolyte solutions is observed to be in the order:

$R_{ZnSO4} > R_{CuSO4} > R_{ZnCl2} > R_{CuCl2}$. Due to adsorption of anions from electrolyte solution, the active layer of the membrane develops a negative surface charge.

Chitosan/polyethersulfone composite membrane was prepared from casting chitosan solution on PES substrate membrane. The Substrate membrane was prepared by phase inversion technique using PES and dimethylacetamide as solvent with and without the addition of polyvinylpyrrolidone (PVP) as pore-forming agent. The effects of the composition of the casting solution on membrane morphology and water permeation were investigated. The membrane prepared from 15 wt % PES with 2.25 wt % PVP demonstrated better water permeability compared to other compositions. CS/PES composite membrane flux and retention were 5.2 l/(m^2 h) and 76.15%, respectively. The mean pore size of the composite membrane was calculated as 0.99 nm.

Membrane processes are gaining importance in water applications as a result of the advances in membrane technology and the increasing requirements on water quality. In this work, 2-hydroxypropyltrimethyl ammonium chloride chitosan with positively charged character and good membrane-forming ability was utilized to fabricate the functional layer of the composite NF membrane. Polyetherimide ultrafiltration membrane was used as the support layer for its excellent thermal and solvent resistance. Effects of polymer concentration, reaction time, cross-linking agent concentration, and cross-linking temperature on membrane performance were studied in detail. When the composite membrane was prepared under optimized conditions and tested at 0.3 MPa and 20 °C, the flux of the composite NF membrane was about 10.9 L/(m^2 h) and the $MgCl_2$ rejection of it was about 83.1%. The surface morphologies of the composite membrane and substrate membrane were observed by scanning electron microscopy. The composite membrane showed a classical positively charged membrane character which had higher rejection to multivalent cations.

Chitosan was modified with a chiral compound and a positively charged compound. Series of novel composite NF membranes were prepared by over-coating the polysulfone UF membrane with the mixture of chitosan and chitosan derivative. The chiral compound, the positively charged compound and their chitosan derivatives were characterised by FTIR and polarimeter. The structure of the membrane was characterized by SEM. The performance of membrane was strictly related to the chiral compound and the positively charged compound grafted to chitosan and its composition. The rejection of P_{2-7} composite NF membrane reached the maximum of 95.2%, and the flux remained to be as high as 687·4 L/(m^2 h) at 0·4 MPa with 1000 mg/L $CaCl_2$. Electrostatic effect had no effect on NaCl, while excellent effect on $CaCl_2$. It was typical positively charged NF membrane, which was suitable for separating the multivalent cation solutes from the feed solution.

Novel positively charged organic–inorganic hybrid ultrafiltration membranes with adjustable charge density were fabricated from blends of water soluble PVA and methylated *N*-(4-*N*, *N*-dimethylaminobenzyl) chitosan (TMBC) in combination with TEOS silica precursor by the sol–gel method and precipitation in a mixture of methanol and water (80 wt %: 20 wt %).

The porous hybrid membrane morphologies, structures, charge and surface properties were characterized comprehensively using SEM, FTIR in the attenuated total reflection mode, outer surface zeta potential and contact angle measurements. The results confirmed that the fabricated membranes were porous, hydrophilic and mildly charged in nature. The water flux and flux recovery ratio (*i.e.* protein fouling resistance) of the membranes were highly dependent on the fraction of TMBC. The protein transmission as a function of pH and the fraction of TMBC was studied for two model proteins (ovalbumin; OVA and lysozyme; LYZ) and found to be controlled by size exclusion and the membrane charge density (controlled by the fraction of TMBC). The highest transmission of proteins at their isoelectric points was obtained for the membrane with 40 wt% of TMBC. The best separation of LYZ from OVA in the model mixture solution was obtained at pH = 11 when membrane A-40 was used in ultrafiltration of protein solution at 2 bar applied transmembrane pressure.

A novel *O*-(carboxymethyl)-chitosan (OCMC) NF membrane is developed via surface functionalization with GO nanosheets to enhance desalting properties (Wang et al. 2015). Using ring-opening polymerization between epoxy groups of GO nanosheets and amino groups of OCMC active layer, GO nanosheets are irreversibly bound to the membrane. The OCMC NF membranes surface-functionalized with GO nanosheets are characterized by FTIR, X-ray photoelectron spectroscopy, SEM, atomic force microscopy, contact angle analyzer, and zeta potential analyzer. The membranes exhibit not only higher permeability but also better salt rejections than the pristine membranes and the commercial NF membranes; besides, the desalting properties are enhanced with the concentration of GO nanosheets increasing. Furthermore, the transport mechanism of GO–OCMC NF membranes reveals that the nanoporous structure of GO–OCMC functional layer and size exclusion and electrostatic repulsion of water nanochannels formed by GO nanosheets lead to the membranes possessing enhanced desalting properties.

Chitosan was blended with copolymer PDMCHEA made from 2-methacryloyloxy ethyl trimethylammonium chloride (DMC) and 2-hydroxyethyl acrylate (HEA), and the blend positively charged nanofiltration membranes (BPCNFMs) were prepared via chemical cross-linking method.

Chemical compositions and structures of BPCNFMs were characterized by attenuated total reflectance FTIR, WAXS, DSC, and field emission SEM, respectively. Mechanical properties of the BPCNFM25 loaded with 25 wt % PDMCHEA were greatly improved, showing 2.4 times higher breaking elongation and 2.0 times higher tensile strength as compared with that of the pristine CS membrane. The surface hydrophilicity and separation performances of BPCNFMs were examined by water contact angle and nanofiltration tests. BPCNFMs were subjected to the nanofiltration, and the effects of PDMCHEA content and feed concentration on nanofiltration performances were investigated. It is found that a high water permeability and salt selectivity, e.g., J_{H2O} = 20.6 L/(m^2·h), R_{ZnCl2} = 97.5%, R_{NaCl} = 57.1% was obtained for the BPCNFM50 containing 50% PDMCHEA (testing with aqueous 1 g/L inorganic salts solutions at 25 °C and 0.6 MPa.

The anti-alga properties and anti-bacteria effects of composite nanofiltration (NF) membranes prepared from sulfated chitosan (SCS) and *N*, *O*-carboxymethyl chitosan (NOCC) were investigated in this study. The base membranes, polyacrylonitrile (PAN) and polysulfone (PS) UF membranes, were used to be controls. Compared with the controls, the adsorptions of the alga on the composite NF membranes were less severe. It suggested that the SCS and NOCC composite NF membranes have anti-alga and antifouling abilities. The chosen bacteria were escherichia coli, bacillus subtilis, staureus, penicillium chrysogenum, and streptomyces jinyangensis. By comparing the colony diameters of different bacteria on various membranes and the growth of bacteria after different time periods, the qualitative conclusions of the anti-bacterial effects of the membranes were drawn. It suggested that all the investigated membranes have some anti-bacterial effects on the five kinds of bacteria and the anti-bacterial effects are related to the active layer material of the composite NF membrane and the cross-linking agent.

The aim of this research was to determine the role of surface charge alteration on the hemocompatibility of chitosan, by modifying the surface charge of chitosan backbone. The modification was achieved by adding negative charges to the chitosan backbones via carboxylation using monochloroacetic acid to produce *N*- and *N*, *O*-carboxymethylchitosan (N and N, O-CMC). The modified material should have carboxymethyl substituent on both the amine and hydroxyl groups, producing negatively charged carboxyl groups on the outer surface. Thus, it is also expected that protein adsorption on the membrane surface would be reduced due to electrical repulsion. Moreover, the ability of the resulted carboxyl group to form stronger hydrogen bonds should increase the membrane selectivity towards the target molecule. Then *N*-, and *N, O*-CMC was blended with PVA to improve the mechanical strength of the membranes, so that the membrane would have better permeability for low-molecular weight compounds. This novel membranes exhibited good permeability properties for small molecules as well as a decrease in protein adsorption on the membrane surface. The structure and the morphology of the resulting membranes were characterized by FTIR, NMR, and elemental analysis.

A chitosan/cellulose acetate composite membrane is prepared by Ismail et al. . The effect of varying cellulose acetate concentration on membrane morphology and performance is studied by using scanning electron microscopy and the composite membrane is characterized by differential scanning calorimetry and thermal gravimetric analysis. Molecular weight cut-off of the composite membrane is found to be 830 Da, which is in the range of nanofiltration. The rejection for copper from a common effluent treatment plant wastewater is observed to be 81.03% at 506.5 kPa applied pressure. The mean pore size is calculated to be 0.78 nm.

Water treatment industries are exploring the possibility to use environmental friendly chemicals and to discover the potential of advanced treatment technology in order to achieve sustainable development. Hybrid coagulation-membrane process

has been introduced and proven to be a reliable water treatment process. In this study, the potential of chitosan as natural coagulant in hybrid coagulation-NF membrane process was studied.

Nanofiltration filtration membranes are prepared with non-biodegradable petrochemical materials. This process is harmful to the ecosystem and consumes a large amount of non-renewable energy. In this study, biodegradable and biocompatible antibacterial cellulose/chitosan NF membranes (BC/CS-NFMs) were fabricated and characterized for their mechanical strength, antimicrobial activity, salt and dye filtration performance, and polyethylene glycol (PEG) retention using TGA, FE-SEM, FT-IR, and XRD. The BC/CS-NFMs were obtained by the hydrolysis and carboxymethylation of dense cellulose/chitosan membranes (BC/CSMs). The tensile strength of the BC/CS-NFMsdecreased as the chitosan content increased. In addition, the thermal stability and antibacterial ability of the BC/CS-NFMs improved. The pore size is less than 1 nm, and a spongy, layered structure is observed in the cross-sectional FE-SEM images. FT-IR analysis shows that a part of the hydroxyl in cellulose transforms to carboxymethyl during the hydrolysis and carboxymethylation of the BC/CSMs. No obvious changes can be observed in the cellulose and chitosan after the blend membrane formation from the XRD measurements. Based on the experimental results on the permeation and rejection of BC/CS-NFMs, different proportions of cellulose and chitosan nanofiltration membranes almost did not affect the water flux and rejection rate. The BC/CS-NFMs showed better water flux and a higher rejection rate in aqueous dye-salt solutions.

A novel positively charged NF membrane was fabricated by incorporating metal-organic frameworks (MOFs) into chitosan polymeric matrix for enhanced removal of multivalent cations. The synthesized MOFs, NH_2-MIL-101(Al) and NH_2-MIL-101(Cr), could be homogeneously dispersed in the chitosan polymeric matrix. The morphologies of MOFs had a significant influence on the permeability of NF membranes. NF membrane filled with NH_2-MIL-101(Al) with rod-like structure attained two times higher flux but similar rejection compared with that filled with NH_2-MIL-101(Cr) with grainy structure. The effect of MOF loadings (NH_2-MIL-101(Al)) on the membrane performance were evaluated by XRD, FTIR and SEM. These positively charged MOF/chitosan NF membranes were able to reject up to 93.0% of $MgCl_2$, and the salt rejection followed the order of $MgCl_2 > CaCl_2 > NaCl > Na_2SO_4$.

To improve the performance of NF membranes, a chiral mesogenic compound, a positively charged compound, and a negatively charged compound were grafted to chitosan, respectively. Series of novel composite NF membranes were prepared by over-coating the polysulfone ultrafiltration membrane with the mixture of chitosan and modified chitosan. The chiral mesogenic compound, the positively charged compound, the negatively compound and their chitosan derivatives were characterized by IR, DSC, polarized optical microscope; the structure of the membrane was characterized by SEM. The performance of composite NF membranes was strictly

related to the novel compounds grafted to chitosan and its composition. The rejection reached the maximum of 95.7 for $CaCl_2$ with P_{2-7} composite NF membrane, corresponding flux was 3155 L/(m^2/h). The rejection reached the maximum of 93 for Na_2SO_4 with P_{3-5} composite NF membrane, corresponding flux was 3879 L/(m^2/h). Comparing with conventional NF membranes, the membranes were used in low pressure with high flux, especially for the separation of high-valence ions from solution. The membranes were typical charged NF membranes.

Carboxymethyl chitosan-zinc oxide bionanocomposite incorporation into the PVDF membrane matrix was prepared by the phase inversion method. The synthesized carboxymethyl chitosan-zinc oxide was confirmed by FTIR, XRD and HRTEM analysis. The modified bionanocomposite membrane was structurally characterized. The modified membranes exhibited low flux than the pure membrane as the membrane morphology overruled it. The hydrophilic water contact angle and the low surface roughness were favourable to mitigate fouling. Inorganic salt rejections were higher for the composite membranes than the pure membrane showing the order as: $Na_2SO_4 > MgSO_4 > NaCl > MgCl_2 > LiCl$. Using humic acid as the model foulant, the fouling was mitigated excellently as the nanocomposite composition incremented on the membrane surface. The modified membranes were able to give the highest flux recovery ratio with least irreversible fouling ratio. The modified membranes also offered superior mechanical and thermal stability and attributed good durability for two repeated cycles.

Carboxymethyl chitosan without a crosslinking agent was blended with polyvinylidene fluoride using non solvent induced phase inversion process. The material was synthesized using monochloroacetic acid and isopropanol and its structural elucidation was done. Synthesized carboxymethyl chitosan was incorporated with PVDF and investigated for its casting dope viscosity, x-ray photoelectron spectroscopy, thermal gravimetric analysis, mechanical properties, contact angle, SEM, atomic force microscopy and filtration studies. The designed precipitation kinetics was reflected on these composite membranes by having reduced pore sizes and increased thickness. MWCO was in the range between 600 and 2000 for the composite membranes. Low surface roughness and contact angle favoured the antifouling nature. Irreversible fouling minified as the carboxymethyl chitosan composition increased on the membrane and exhibited excellent mechanical and thermal stability and flux recovery ratio. The membranes were durable after two repeating cycles.

Phosphorylated chitosan (PCS) with tailored amount of phosphate groups was synthesized, and novel composite NF membranes (NFMs) with tunable surface charges were prepared by coating PCS onto polyacrylonitrile (PAN) supporting layer and subsequently cross-linked by glutaraldehyde (GA). Chemical structures and compositions of PCS and NFMs, along with morphologies, surface charges, and performance of NFMs were represented by using FTIR, TG, ATR-IR, SEM-EDX, AFM, streaming potential analyzer, and cross-flow flat permeation test, respectively. The effect of different phosphorous abundance of PCS precursors on the surface charge and permselectivity of NFMs was investigated systematically. It was illustrated that the incorporation of phosphate group with high-substitute degree

did enhance the surface charge and selectivity of NFM, respectively, while retaining its permeability. The resultant membrane showed a zeta potential of −77.6 mV at 1 mol/m^3 KCl electrolyte solution and pH 7.0, significantly more superior than that of the commercial DL membrane. For the feedwater (Na_2SO_4 + $MgCl_2$, 1.0 g/L), the mass ratio of SO_4^{2-}/Cl^- and Mg^{2+}/Na^+ decreased from initial 0.5:1 in the feed to 1.58×10^{-2} and 0.254 in the permeate after filtration by the optimal NF membrane. Anionic dyes removal tests also confirmed the existence of negative charge characteristics from the prepared membranes, and pivotal role of the Donnan exclusion in separating performance. In addition, NFM4 exhibits good chemical stability in the long-term operation (Ghaemi et al. 2018).

5.3.4 Technology of Ultrafiltration

Chitin ultrafiltration membranes were prepared by the wet method using DMA/NMP/LiCl as the casting solvent and water as the gelation medium (Uragami et al. 1981). These membranes were asymmetric porous structures, and the ultrafiltration characteristics for an aqueous solution of polyethylene glycol 6000 were significantly influenced by the temperature during membrane preparation. These results are attributed to the absorption of water from the atmosphere into the casting solutions during the casting process. Because DMA and NMP are solvents with very low volatility and very high hygroscopicity and since LiCl is deliquescent, the resulting membrane structures are affected by the temperature during membrane preparation. In the preparation of the chitin membranes, if the gelation medium was changed from water to 2-propanol, permeability for low-molecular-weight solutes and tensile strength of the chitin ultrafiltration membranes improved.

To obtain ultrafiltration membranes for blood treatment, as dried quaternized CS membranes were immersed into ethylene glycol diglycidyl ether (EGDGE) containing a small amount of NaOH, the hydroxyl groups in the quaternized CS molecules were cross-linked with EGDGE and water-insoluble quaternized CS membranes were obtained. When these water-insoluble membranes were again immersed in an aqueous solution of sodium heparin, complex between quaternized CS and sodium heparin were formed, enabling the preparation of heparinized CS membranes. As can be seen 13 these heparinized CS membranes could perfectly permeate low-molecular-weight solutes such as urea, creatinine and vitamin B_{12} and completely block protein such as albumin; they also showed an excellent antithrombogenicity in an *in vivo* test (Uragami et al. 1988).

Chitosan membranes for ultrafiltration were prepared by casting acetic acid solutions of chitosan including an each amount of polyethylene glycol (PEG) as additives. Additives were eliminated by elution in hot water after the neutralization of membrane. The water flux of the membrane was measured and the membrane structure was observed by the scanning electron microscope. The membrane was found to consist of three layers; the surface, the internal microporous layer and the opposite

surface. With increasing the amount of PEG from 25 phr to 100 phr at the membrane preparation, the water flux increased largely more than 1000 folds, but there was only a 10% increase in the degree of hydration, H, of the membrane. The drastic change in the macroscopic membrane structure occurred in the vicinity of $H = 0.7$, i.e., the micropores which penetrated throughout the membrane were formed in the region $H > 0.7$. The molecular weight of PEG also affected the membrane structure and the water flux.

Chitosan membranes for ultrafiltration were prepared by casting acetic acid solutions of chitosan including PEG as additive. The additive was eliminated by elution in hot water after the neutralization of membrane. The ultrafiltration through the chitosan membrane was carried out using the solutions of proteins such as bovine serum albumin and γ-globulin. It was found that the rejection of proteins depended on the pH of the solution and it changed drastically in the vicinity of the pH of the isoelectric point of the protein (pH_I). The high rejection was observed at the pH lower than the pH, and the rejection was no longer observed beyond the pH_I. These facts mean the interaction between the membrane charge and the protein.

The permeation rate of the solution was kept constant in the pH region more than pH 6 but it largely decreased in the lower pH region. It was considered from the SEM observations that the chitosan membrane was partially dissolved in the low pH buffer solution and at the same time some micropores which had existed on the air-side surface disappeared and became the dense layer.

In addition, it was recognized that an appropriate membrane structure was required for the charged ultrafiltration membrane.

Macroporous chitosan membranes with controlled pore sizes and good mechanical properties were prepared and cross-linked with ethylene glycol diglycidyl ether to increase their chemical stability.

Because of their amine groups, they can serve as anion-exchangers (with an ionexchange capacity as high as 0.83 meq/g dry cross-linked membrane) and can be employed for protein separations in the ion-exchange mode. At pH < 7, their surface is positively charged, and they can adsorb proteins with a pI<6 at appropriate pHs. Five proteins, namely ovalbumin (pI = 4.6), human serum albumin (pI = 4.8), soybean trypsin inhibitor (pI = 4.5), lysozyme (pI = 11) and cytochrome C (pI = 10.6) were selected as model proteins to investigate their adsorption on the chitosan membranes. Relatively high dynamic capacities were achieved at a flow rate of 2 ml/min, namely 11.6, 19 and 20.8 mg/ml membrane for human serum albumin, ovalbumin and soybean trypsin inhibitor, respectively. These proteins could be efficiently recovered (91–98%) from the membranes using a 1 N NaCl in 0.02 N sodium phosphate solution (pH 6) as eluant. Protein separations were performed from binary mixtures (ovalbumin–lysozyme, human serum albumin–cytochrome C, and soybean trypsin inhibitor–cytochrome C), and high purity products (∼99%) obtained in a single pass. These membranes showed high stability and reproducibility.

Formed-in-place ultrafiltration membranes were formed from dilute solutions of chitosans with different molecular weights in 1% acetic acid on a macroporous titanium dioxide substrate. The ultrafiltration properties were characterized by

investigating the rejection and permeability of a 1.0 g/L BSA solution at various pH and ionic strength conditions. The membrane stability to the crossflow shear and to the ionic strength was investigated. There was very little dependence of the membrane-formation capability and the membrane properties on the chitosan molecular weight. In contrast, pH had a marked effect on membrane surface properties, membrane stability, and membrane morphology (Wang and Spencer 1998).

The resistance of novel surface crosslinked chitosan/poly(acrylonitrile) composite nanofiltration membranes to pH and organic solvents was studied with respect to the effects of crosslinking parameters, namely, glutaraldehyde concentration and crosslinking time. The pH resistance was determined by permeation of aqueous acidic (pH 2.5) and basic (pH 11) solutions as well as swelling studies in the pH range of 2.5–11. The solvent resistance was determined by swelling, immersion, and permeation studies with several industrially important organic solvents, namely methanol, ethanol, iso-propanol, methyl ethyl ketone, ethyl acetate and hexane. It was observed that the crosslinked composite membranes maintain the permeate fluxes for test solvents for 2 h of continuous operation without any significant change in flux. SEM studies on membrane samples after immersion as well as permeation with the above-mentioned solvents indicated that the membrane morphology was maintained. The results are explained in terms of solvent–membrane polar and hydrophobic interactions, using solubility parameters of membrane and solvents and dielectric constants of solvents. Pure water flux and polyethylene glycol transmission data indicated that at pH 2.5 and 11, the membrane stability increased with increasing glutaraldehyde concentration and was much better at pH 11 than at pH 2.5. All surface crosslinked membranes showed reduced swelling between pH 4–10 (Musale and Kumar 2000).

The fabrication of chitosan based ceramic membranes using dip coating technique is reported by Jana et al. Low-cost ceramic supports were prepared from local clay of IIT Guwahati and kaolin with an average pore size of 1093 nm and porosity of 0.37. Different ceramic membranes were prepared by varying chitosan concentration and dipping time and were characterized using SEM, air and hydraulic permeability tests. The average pore sizes were in the range of 760–13 nm which confirmed that the chitosan impregnated ceramic membranes were applicable for both MF and UF applications. An increase in both chitosan concentration and dipping time was found to reduce the pore size. The lowest pore size ultrafiltration membrane (pore size: 13 nm) was used for the removal of mercury and arsenic from wastewater by polymer enhanced ultrafiltration (PEUF) technique using PVA as chelating agent. The effects of initial concentrations of mercury, arsenic and PVA on the extent of removal of both the heavy metals were investigated in detail. The efficiency of PEUF was explored in terms of rejection of metals and permeates flux. Almost 100% removals were observed for both 500 μg/L mercury and 1000 μg/L arsenic.

Liquids filtration technology as one of the paths to sustainable water use is getting more and more attention. A new type of high flux ultrafiltration or nanofiltration medium based on electrospun fibrous scaffold and ultrathin top barrier layer was fabricated recently. Based on this new method, the chitosan which is one of the best

top layer materials due to its hydrophilicity and high water-permeability was coupled with electrospun polyvinylidene fluoride (PVDF) nanofibers to compose a new type UF membrane. In this work, the chitosan was crosslinked and modified by glutaraldehyde (GA) and terephthaloyl chloride (TPC) to adjust its water resistance and surface properties. The modified membrane was characterized by FTIR, SEM, UV-spectra, static water contact angle analysis and filtration test. The modified membrane gets broader operating environment range and keeps a good flux rate and rejection efficiency in bovine serum albumin filtration tests at 0.2 MPa, about 70.5 L/(m^2 h), rejection efficiency >98% which are higher than that of 57.1 L/(m^2 h), rejection efficiency∼98% of the commercial UF membranes, while the fouling of the membrane was kept at a very low level. This work may provide a practical possibility to the water filtration industry (Zhao et al. 2012).

Chitosan was successfully grafted onto the top surface of a bromomethylated poly(phenylene oxide) (BPPO) ultrafiltration membrane without pretreating the membrane at harsh conditions and/or using other cross-linkers. Due to the grafting of polar groups of chitosan onto the membrane top surface, the hydrophilicity of the membrane top surface and the polar component of the total surface energy are improved compared to the pristine BPPO membrane. Theoretical calculation shows that the polar component of surface energy is a major contributor to the reduction in interfacial free energy of the membrane surface and the interaction strength between foulants and membrane surface. Therefore, the foulants adsorbed onto the top surface of chitosan/BPPO composite membranes are much easier to desorb during the cleaning process and as a result, a higher flux recovery was obtained compared to the pristine BPPO membrane. Moreover, due to the antibacterial nature of chitosan, such composite membranes show a better antibacterial property and the antibacterial rate was improved by 70% in comparison with the pristine BPPO membrane (Feng et al. 2014).

Combing the liquid-phase polymer-based retention, LPR technique with an ultrafiltration membrane facilitates the separation of arsenic ionic species that are retained by the functional groups of hydrophilic polyelectrolytes. Arsenate retention by P(ClAETA) at a high arsenate concentration (47.6 mg/L was 58% and this removal capacity increases gradually, reaching 100% retention when the arsenate concentration in the cell was at minimum (5.5 mg/L) using molar ratio (20:1) polymer: As(V). Arsenic removal was also determined at low concentrations (in μg/L). The results show that P(ClAETA) removes 65% of arsenate at lower concentration and that the arsenate concentration in each 20 mL of filtrate above Z = 3 is below the maximum permissible level of the World Health Organization (WHO). The charge–discharge process shows that the discharge process of the arsenate ions from polymers can be performed when the polymer–arsenate was in contact with acid solution from the reservoir. Removal of arsenic from the Camarones River water was also performed by using P(ClAETA). The water-soluble polymer showed a high performance (100%) for the first Z values and then decreased up to 16% for Z = 10.

Biocompatible and naturally occurring chitosan was used as an additive for the preparation of a polysulfone ultrafiltration membrane. Two different compositions of polysulfone in *N*-methylpyrrolidone (NMP) and chitosan in 1% acetic acid were

blended to prepare PSf–CS ultrafiltration membranes by the diffusion induced phase separation (DIPS) method. The proper blending of polysulfone and chitosan in PSf–CS membranes was confirmed by ATR-IR analysis. The surface and cross-sectional morphology of the membranes was studied by scanning electron microscopy (SEM). The membrane hydrophilicity was determined by water uptake and contact angle measurements. The PSf–CS membrane showed an enhanced hydrophilicity compared to a PSf ultrafiltration membrane. The time dependent permeation studies revealed the improved flux of PSf–CS membranes. PSf–CS membranes were subjected to bovine serum albumin (BSA) protein rejection studies. An improved antifouling property was observed for PSf–CS blend membranes as compared to pristine PSf ultrafiltration membranes. Both the permeation and antifouling properties of PSf–CS membranes increased with an increase in chitosan composition.

Membrane processes are gaining importance in water applications as a result of the advances in membrane technology and the increasing requirements on water quality. In this work, 2-hydroxypropyltrimethyl ammonium chloride chitosan with positively charged character and good membrane-forming ability was utilized to fabricate the functional layer of the composite nanofiltration membrane. Reinforced polyetherimide ultrafiltration membrane was used as the support layer for its excellent thermal and solvent resistance. Effects of polymer concentration, reaction time, cross-linking agent concentration, and cross-linking temperature on membrane performance were studied in detail. When the composite membrane was prepared under optimized conditions and tested at 0.3 MPa and 20 °C, the flux of the composite NF membrane was about 10.9 L/(m^2 h) and the $MgCl_2$ rejection of it was about 83.1%. The surface morphologies of the composite membrane and substrate membrane were observed by scanning electron microscopy. The composite membrane showed a classical positively charged membrane character which had higher rejection to multivalent cations.

Polyethersulfone nanofiltration membranes were prepared by blending of *O*-carboxymethyl chitosan CC)/Fe_3O_4 nanoparticles via the phase inversion method. CC bound Fe_3O_4 magnetic nanoparticles (CC–Fe_3O_4 NPs) were synthesized by the binding of CC onto the surface of Fe_3O_4 magnetic nanoparticles via co-precipitating method. The modified membranes had considerably higher pure water flux and permeation compared to the unfilled PES membrane. Surface hydrophilicity of the modified membranes was improved due to water affinity improvement of the membrane surface. However, high content of CC–Fe_3O_4 NPs (> 0.5%) in the casting solution diminished membrane performance due to agglomeration of the nanoparticles in the polymer matrix. The antifouling properties of the membranes were evaluated by powder milk solution and measuring the pure water flux recovery ratio (*FRR*). The 0.5 wt % CC–Fe_3O_4 NPs–PES membrane exhibited the highest *FRR* value (91.7%) and the lowest irreversible fouling resistance (R_{ir}) value (8.33%). Nanofiltration performance was also examined by retention of Direct Red 16 dye. As a conclusion, the addition of CC–Fe_3O_4 NPs at lower concentration could result in membranes with superior pure water flux, higher rejection and good fouling resistance compared to the unfilled PES membrane.

Chitosan was successfully grafted onto the top surface of a bromomethylated poly(phenylene oxide) (BPPO) ultrafiltration membrane without pretreating the membrane at harsh conditions and/or using other cross-linkers. Due to the grafting of polar groups of chitosan onto the membrane top surface, the hydrophilicity of the membrane top surface and the polar component of the total surface energy are improved compared to the pristine BPPO membrane. Theoretical calculation shows that the polar component of surface energy is a major contributor to the reduction in interfacial free energy of the membrane surface and the interaction strength between foulants and membrane surface. Therefore, the foulants adsorbed onto the top surface of chitosan/BPPO composite membranes are much easier to desorb during the cleaning process and as a result, a higher flux recovery was obtained compared to the pristine BPPO membrane. Moreover, due to the antibacterial nature of chitosan, such composite membranes show a better antibacterial property and the antibacterial rate was improved by 70% in comparison with the pristine BPPO membrane (Feng et al. 2014).

Heavy metal rejection properties of chitosan based polysulfone/chitosan (PSf/CS), polysulfonef/*N*-succinyl chitosan (PSf/NSCS) and polysulfone/*N*-propylphosphonyl chitosan (PSf/NPPCS) ultrafiltration membranes were evaluated. The rejection of membranes towards the copper, cadmium and nickel ions was studied during ultrafiltration by polymer enhanced ultrafiltration (PEUF) processes. The flux change during the UF process and the effect of pH on the rejection were determined. The membrane recycling property was studied during PEUF process by filtering chelated $CuSO_4$ solution. A maximum of 78% of Cu, 73% of Ni and 68% of Cd rejection for M-5 membrane, 75% of Cu, 71% of Ni and 66% of Cd rejection for M-8 membrane and 76 of Cu, 69% of Ni and 66% of Cd rejection for M-2 membrane with reasonably good flux was observed. Further improvement in heavy metal ion rejection was achieved by PEUF process. Membrane M-5 showed a maximum of 98%, 95% and 92% rejection for Cu, Ni and Cd respectively with steady state flux of 117 L/m^2 h. An increase in membrane recycling property after the metal ion rejection was mainly attributed to the hydrophilicity of CS, NSCS and NPPCS.

A sample of *N,N,N*-trimethylchitosan chloride (TMC) was prepared and its ability to remove arsenate and chromate from an aqueous solution was evaluated. The removal capacity was quantified by a liquid-phase polymer-based retention system. A washing method was used to determine the effect of pH, molar ratio, contact time, and interfering ions on the removal of the anions. The results showed that TMC exhibits a high affinity for the divalent species $HAsO_4^{2-}$ and CrO_4^{2-} because the majority of the retention capacity occurred at pH values between 6 and 10. A removal maximum was found for experiments with a 10:1 molar ratio and pH 8.0, achieving a 73.0% removal of As(V) and a 94.0% removal of Cr(VI). The evaluation of the effect of the contact time of the polymer–SR (species to remove) solution prior to diafiltration revealed that the interaction between TMC and the anions is produced rapidly. Furthermore, TMC selectivity in the presence of interfering ions, such as chloride and sulphate, and maximum retention capacity were evaluated.

Finally, permeate flux behaviour was briefly discussed under different conditions of removal.

CS was successfully grafted onto the top surface of a bromomethylated poly(phenylene oxide) (BPPO) UF membrane without pretreating the membrane at harsh conditions and/or using other cross-linkers (Feng et al. 2014).

Due to the grafting of polar groups of CS onto the membrane top surface, the hydrophilicity of the membrane top surface and the polar component of the total surface energy are improved compared to the pristine BPPO membrane. Theoretical calculation shows that the polar component of surface energy is a major contributor to the reduction in interfacial free energy of the membrane surface and the interaction strength between foulants and membrane surface. Therefore, the foulants adsorbed onto the top surface of CS-BPPO composite membranes are much easier to desorb during the cleaning process and as a result, a higher flux recovery was obtained compared to the pristine BPPO membrane. Moreover, due to the antibacterial nature of CS, such composite membranes show a better antibacterial property and the antibacterial rate was improved by 70% in comparison with the pristine BPPO membrane.

N-phthaloyl chitosan (NPHCs), which could be dissolved in various organic solvents, is synthesized for the modification of poly (ether imide) (PEI) ultrafiltration membrane. Blend membrane with 2 wt % NPHCs exhibited higher pure water flux (112.2 l/(m^2 h), higher water content (63.4%) and lower hydraulic resistance (3 kPa/l/(m^2 h). The top surface morphology of the control PEI membrane changed from a dense surface to visible pores with the increase in NPHCs concentration. The surface roughness of PEI membranes increased with an increase in NPHCs concentration in the casting solution. Application studies were carried out to find the rejection and permeate flux of proteins such as bovine serum albumin (BSA), egg albumin (EA), pepsin and trypsin and toxic heavy metal ions such as Cr(III), Zn(II), Cd(II) and Pb(II). The result shows that the flux and separation performances are dependent upon the content of NPHCs. Furthermore, the blend membranes were subjected to the determination of pore statistics and MWCO. It was found that the blending of NPHCs into the PEI membrane had a visible effect upon MWCO and pore size. The significant effect of hydrophilicity of NPHCs on the fouling of PEI/NPHCs blend membranes by BSA was also discussed. It was found that the blend membranes with 2 wt % NPHCs content had a higher FRR (88.6%), higher reversible fouling (23.7%) and lower irreversible fouling (11.4%) which explained their improved antifouling properties. Thus, the modified chitosan proved to play an important role in the improvement of UF membrane performance.

Carboxymethyl chitosan without a crosslinking agent was blended with polyvinylidene fluoride (PVDF) nonsolvent induced phase inversion process. Carboxymethyl chitosan was synthesized using monochloroacetic acid and isopropanol and its structural elucidation was done. Synthesized carboxymethyl chitosan was incorporated with PVDF and investigated for its casting dope viscosity, x-ray photoelectron spectroscopy, thermal gravimetric analysis, mechanical properties, contact angle, SEM, atomic force microscopy and filtration studies. The designed precipitation kinetics was reflected on these composite membranes by having

reduced pore sizes and increased thickness. MWCO was in the range between 600 and 2000 for the composite membranes. Low surface roughness and contact angle favoured the antifouling nature. Irreversible fouling minified as the carboxymethyl chitosan composition increased on the membrane and exhibited excellent mechanical and thermal stability and flux recovery ratio. The membranes were durable after two repeating cycles.

5.3.5 Technology of Microfiltration

An amphoteric chitosan derivative, containing carboxymethyl (-COOH) groups and amine (-NH_2) groups, had been prepared. FT-IR spectra confirmed the presence of γ_{sym}COO (1408 cm^{-1}) and γ_{as}COO (1584 cm^{-1}) on the structural units of the chitosan derivative, and ^{13}C NMR spectra revealed that carboxy methylation reactions took place not only on the -OH but also on the -NH_2. N, O—carboxymethyl chitosan (CM—CS)/poly(ethersulfone) (PES) composite microfiltration (MF) membranes were prepared by immersing PES MF membranes into CM—CS solutions and cross-linking with glutaraldehyde. Streaming potential measurements indicate that the CM—CS/PES composite MF membranes possess a weaker positively charged characteristic at low pHs but a stronger negative charged characteristic at high pHs than the chitosan composite membrane (CS/PES). It was further observed that the CM-CS/PES composite membranes have higher adsorption capacities of bovine serum albumin (BSA) than the CS/PES composite membranes and PES membrane at lower pH 3.0–4.7, and lower adsorption capacities at higher pH 6.0–8.0. Therefore, the CM-CS/PES composite membranes may be suitable for resistant to protein fouling at high pHs or protein adsorption separations at low pHs applications.

An amphoteric CS derivative, containing carboxymethyl (-COOH) groups and amine (-NH_2) groups, had been prepared by Zhao et al. (2002).

FTIR spectra confirmed the presence of ysymCOO (1408 cm^{-l}) and ysymCOO (1584 cm^{-1}) on the structural units of the CS derivative, and ^{13}C NMR spectra revealed that carboxymethylation reactions took place not only on the -OH but also on the -NH_2. *N,* O-carboxymethyl chitosan (CM-CS)/poly(ethersuIfone) (PES) composite MF membranes were prepared by immersing PES MF membranes into CM-CS solutions and cross-linking with glutaraldehyde. Streaming potential measurements indicate that the CM-CS/PES composite MF membranes possess a weaker positively charged characteristic at low pHs but a stronger negative charged characteristic at high pHs than the chitosan composite membrane (CS/PES). It was further observed that the CM-CS/PES composite membranes have higher adsorption capacities of BSA than the CS/PES composite membranes and PES membrane at lower pH 3.0–4.7, and lower adsorption capacities at higher pH 6.0–8.0. Therefore, the CM-CS/PES composite membranes may be suitable for resistant to protein fouling at high pHs or protein adsorption separations at low pHs applications.

Modification of hydrophobic membrane by chitosan solution for the purpose of reducing protein fouling was investigated.

The membrane used was flatsheet polyvinylidenefluoride (PVDF) of 0.22 μm pore size. The membranes were modified by 3 different methods, i.e. immersion method, flow through method and the combined flow through and surface flow method. Chitosan solution concentration and modification time were varied. The modified membranes were then neutralized with NaOH solution. The results of scanning electron SEM and FTIR study of modified membranes compared to unmodified membranes confirmed that there was chitosan coated on the membrane surfaces. The water contact angles and water fluxes decreased with increasing chitosan concentration and modification time. The result also indicated that modified membranes had higher hydrophilicity than unmodified membrane. In protein fouling experiment, BSA was used as a protein model solution. Modified membranes exhibited good anti-fouling properties in reducing the irreversible membrane fouling. The membrane modified by a combined flow through and surface flow method showed the best anti-fouling properties compared with other methods. Protein adsorption on the modified membrane was highest at the isoelectric point (IEP) of BSA solution and decreased as the solution pH was far from the IEP (Wang et al. 2013).

The removal of endotoxin from water using a positively charged microfiltration membrane was investigated. The membrane was prepared by crosslinking a chitosan coating layer with glutaraldehyde vapor on a cellulose microporous substrate. The positively charged CS/CA membrane showed promising removal efficiency of endotoxin at a high water flux.

Novel thin film composite (TFC) membrane was prepared by organoclay/chitosan nanocomposite coated on the commercial polyvinylidene fluoride (PVDF) microfiltration membrane. The procedure was set to prepare a PVDF/chitosan TFC membrane without applying a cross-linker agent. Various quantities of two different grades of organoclay (Cloisite 15A and 30B) were added into chitosan solution to find the optimum value and to compare the effect of organic modifier of clay nanoparticles on the prepared TFC characteristics. The membranes were examined with aqueous dye solution regarding to the performance qualification. The results showed that formation of coating layer decreased the water flux in all the prepared TFC membranes. Methylene blue dye removal increased with applying organoclay particles in chitosan coating. Cloisite 15A and 30B showed different properties in elimination of various dyes as well as alteration of feed acidity. The morphology of fabricated TFC membranes was compared to each other using SEM. The coating layer containing Cloisite 30B was denser rather than that of Cloisite 15A. X-ray diffraction pattern of prepared nanocomposites containing organoclay and chitosan indicated greater d-spacing for Cloisite 15A rather than 30B. The investigation of functional groups in clay/chitosan nanocomposite coating was performed by FTIR. The chemical structure (functional groups) of prepared TFC membranes offered adsorption as dominating dye removal mechanism.

The removal of endotoxin from water using a positively charged MF membrane was investigated. The membrane was prepared by cross-linking a coating layer with glutaraldehyde vapor on a cellulose microporous substrate. The positively charged

CS/CA membrane showed promising removal efficiency of endotoxin at a high water flux. Nanofibrous filter media of polyamide-6/CS were fabricated by electrospinning onto a satin fabric substrate and characterized by SEM, FTIR, and WCA. Anionic dye removal capability of the filter was investigated for Solophenyl Red 3BL and Polar Yellow GN, respectively, as acidic and direct dyes were investigated with respect to solution parameters (pH and initial dye concentration) and membrane parameters (electrospinning time and ratio) through filtration system. Experiments were designed using response surface methodology based on five-level central composite design with four parameters to maximize removal efficiency of the filter media. Moreover, the effect of parameters and their likely interactions on dye removal were investigated by mathematically developed models. The optimum values for solution pH, initial dye concentration, electrospinning time, and CS ratio were predicted to be 5.50 mg/L, 4 h, 30% and 5.100 mg/L, 4 h,10%, respectively, for achieving 96% and 95% removal of Solophenyl Red 3BL and Polar Yellow GN. Evaluation of the estimation capability of applied models revealed that the models have a good agreement with experimental values. This study demonstrated that polyamide-6/CS nanofibrous membrane has an enormous applicable potential in dye removal from aqueous solutions.

The novel hybrid material, clay/polymer nanocomposite has represented a desired alternative for designing the mixed matrix membranes due to their excellent overall performance, such as good hydrophilic nature, high mechanical strength, enhanced thermal stability, etc. In this study, we report a feasible method to fabricate a novel "loose" nanofiltration membrane by blending with chitosan–monmorillonite (CS–MMT) nanosheets *via* phase inversion method. The results of FT-IR, XRD and TEM showed that CS-MT nanosheets were successfully fabricated. It was found that the hydrophilicity and water flux of hybrid membranes greatly improved after adding CS–MMT nanosheets. Meanwhile, the hybrid membranes exhibited a preferable antifouling property, that is, high flux recovery ratio (ca. 92) and low total flux decline ratio (ca. 0.26) when CS-MT content was only 1.0 wt %. Additionally, CS-MT nanosheets provided an enhanced mechanical property, thus giving rise to significant promotion in both tensile strength and elongation at break of the hybrid membranes. More importantly, the "loose" nanofiltration membrane showed higher rejection for Reactive Black 5 and Reactive Red 49, and lower rejection for bivalent salts unlike the conventional nanofiltration membranes. Therefore, the "loose" nanofiltration membrane could be used for dyes purification with low pressure and high efficiency.

The inherent hydrophilic nature of chitosan has gained significant attention with respect to its application in water treatment processes. Cadogan et al. aimed to synthesize chitosan–glycerol (CSG) membranes for MF applications in wastewater treatment via the solution casting and solvent evaporation technique. CSG membranes were prepared in the ratios 2:1, 3:1, and 4:1 and were cross-linked with phosphoric acid in the presence of ethanol. The synthesized membranes were characterized by tensile strength, swelling, FTIR -attenuated total reflectance and SEM studies to investigate their structural properties. The porosifier glycerol had a great influence on the tensile strength and elongation than the cross-linker. The pore

size distribution of the membranes ranged from 28.1 to 51.6 Å with BET surface areas of 13.8–23.8 m^2/g. Water permeation studies were conducted using a cross-flow MF module in batch analyses. The results indicated that 2:1 CSG membranes effectively removed over 95% of bacteria notably, *Escherichia coli* from wastewater. Various blocking mechanisms were investigated and are discussed in detail utilizing various fouling models such as standard, intermediate, complete, and cake formation, of which the cake formation model had the highest correlation coefficient of these models.

The fabrication of polyvinylidene fluoride (PVDF) membranes was studied using chitosan as a hydrophilic additive for flux enhancement and stability. Chitosan was blended with PVDF at 0.5, 1.0, 1.5, 2.0 and 2.5% (w/w) concentration, to prepare mixed matrix membrane using the phase inversion method. Chitosan presence within the polymer matrix was confirmed using FTIR and SEM. The fabricated membranes were also analyzed for their structural and surface characteristics, including pore size distribution, porosity, contact angle, wettability and surface roughness. The water contact angle of the membranes has decreased upon the addition of chitosan. However, the wettability of the membranes was affected more profoundly due to an increase in their pore size. Microfiltration tests showed that the addition of CS was helpful in the reduction of fouling by BSA. However, membranes with CS content higher than 1.0% endured surface micro-ruptures during the filtration tests due to swelling resulting from high water uptake. This simple method of fabrication of PVDF/chitosan membranes is a potential solution for the low flux and fouling of common PVDF microfiltration membranes. Furthermore, understanding the impact of chitosan on the fabricated membranes can facilitate the fine tuning of the membrane properties to achieve higher efficiencies (Elizalde et al. 2017).

5.3.6 Pervaporation

5.3.6.1 Water-Permselective Membranes

Chitosan, whose deacetylations were 99 (H3F) and 93.5% (L3F), has been investigated for separating water-ethanol mixture by pervaporation. The experiment was carried out at 5–50% water content in 35–70 °C range, Water permeates preferentially through the chitosan membranes. The permeation rate Q of the membranes increases with the increment of water content in the mixture, while its separation factor a has a maximum at the water content of about 25%. The Q value increases as the temperature rises, the degree of the increase being larger for L3F chitosan. Temperature dependence of a is very slight for H3F chitosan, while it decreases with temperature for L3F chitosan and membranes of other materials. Chitosan membranes whose a and Q are better balanced than other membranes were found to be promising for separation of water-ethanol mixture by pervaporation.

Homogeneous and composite chitosan based membranes were prepared by the solution casting technique. The membranes were investigated for the pervaporation dehydration of isopropanol-water systems. The effects of feed concentration and temperature on the separation performance of the membranes were studied. In terms of the pervaporation separation index (PSI), the composite membrane was more productive than the homogeneous membrane for pervaporation of feed with high isopropanol content. It was observed that permeation increased and the separation factor decreased with the temperature. Modification of the homogeneous chitosan membrane by chemical crosslinking with hexamethylene diisocyanate improved the permselectivity but reduced the permeation rate of the membrane.

Chitosan composite membranes were prepared by casting solution onto a porous polyethersulfone ultrafiltration membrane with various surface crosslinking densities. Pervaporation performance of water-alcohol mixtures through the surface crosslinked chitosan composite membranes exhibited a high selectivity value with a low permeation flux. By increasing feed ethanol concentration, permeate flux decreased and water concentration in the permeate decreased drastically at a feed ethanol concentration above 97 wt%. Permeation rate of chitosan composite membranes is less temperature-dependent than that of PVA. IPA-water mixture has a similar tendency as that of ethanol-water mixture in pervaporative dehydration performances (Lee et al. 1991).

Blend membranes of chitosan and *N*-methylol nylon 6 were prepared by solution blending. Their pervaporation performances for the separation of ethanol–water mixtures were investigated in terms of acid (H_2SO_4) post-treatment, feed concentration, blend ratio and temperature. The pervaporation performance of the blend membranes was significantly improved by ionizing with H_2SO_4. The blend ratio of chitosan and *N*-methylol nylon 6 plays a different role at feed solutions of low and high water content. At a feed solution having low water content, an increase in chitosan content caused a decrease in permeability and an increase in separation factor. At a feed solution having high water content, the permeability increases with an increase in chitosan content, while the separation factor shows a maximum value around 60 wt% chitosan. It is proposed that extra permeation channels generated from the phase separation boundary between ionized chitosan and *N*-methylol nylon 6 account for the abnormal temperature dependence of pervaporation performance of the blend membranes.

Chitosan composite membranes having a microporous polysulfone substrate were prepared and tested for the pervaporation dehydration of aqueous isopropanol mixtures. When the composite membrane experienced excessive swelling at the feed mixture of high water content, the composite membranes were found to be segregated in structure due to the opposite characteristics to water of chitosan and polysulfone. Efforts to enhance the structural stability under various pervaporation operational conditions were made. The polysulfone substrate was immersed into hydrophilic binding polymer solutions such as polyvinyl alcohol, polyacrylic acid, and hydroxyethylcellulose before the casting of chitosan layer to increase the affinity between the thin chitosan layer and porous polysulfone layer which resulted in

increased geometrical stability of the chitosan/polysulfone composite membranes. The chitosan layer was crosslinked with glutaraldehyde and H_2SO_4 in acetone solution to control the permselectivity.

For the separation of methanol/MTBE (methyl *tert*-butyl ether) mixtures, methanol selective chitosan composite membranes were prepared and tested for pervaporation experiments. When anionic surfactants are added into the cationic chitosan solution, the solution viscosity was drastically decreased due to the collapsed chain conformation. Pervaporation characteristics of surfactant modified chitosan membrane were substantially improved due to the decreased membrane thickness and possible enhanced affinity to methanol. Rheological data of the casting solution was measured using viscometer and the surface morphology of the surfactant complexed chitosan membrane was investigated by atomic force microscopy (AFM).

Figure 5.14 shows the effects of TEOS content in the *q*-Chito-TEOS hybrid membranes on the normalized permeation rate and the ethanol concentration in the permeate for an azeotrope of ethanol-water during pervaporation. The TEOS content in mole per cent in the figure is for the pyranose ring in the *q*-Chito molecule. The permeate ethanol concentrations of all *q*-Chito-TEOS hybrid membranes were very low compared with that in the feed solution. These results suggest that the *q*-Chito-TEOS hybrid membranes showed high water/ethanol selectivity. High water/ethanol selectivity was observed in the *q*-Chito-TEOS hybrid membranes containing up to 45 mol % TEOS; however, selectivity decreased when the TEOS content was further increased. The best performance was observed for the *q*-Chito-TEOS hybrid membrane with 20 mol % TEOS content, where ethanol could not be detected in the permeate by gas chromatography. This result suggests that ethanol could be rejected almost perfectly by this *q*-Chito-TEOS hybrid membrane. The normalized permeation rate gradually decreased by increasing the TEOS content up to 45 mol %, but it then increased at high, TEOS contents. These results can be

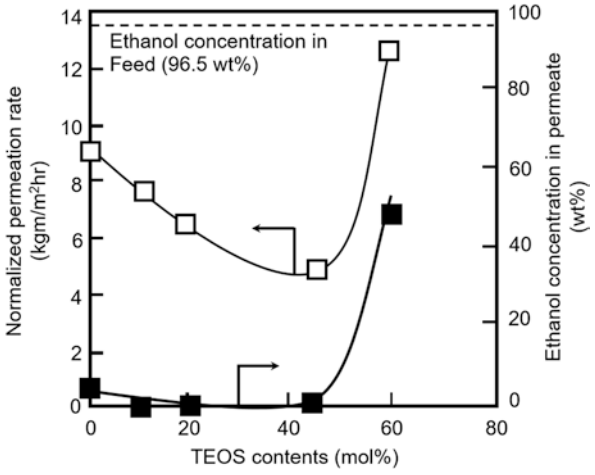


Fig. 5.14 Effects of TEOS content on the normalized permeation rate (□) and the ethanol concentration in the permeate (■) during pervaporation of an azeotrope (96.5 wt%) of ethanol-water through *q*-Chito-TEOS hybrid membranes

attributed to both the formation of a cross-linked structure and a difference in the state of the cross-linked network.

Polyion complex membranes made by blending 84% deacetylated chitosan and sodium alginate biopolymers followed by crosslinking with glutaraldehyde were tested for the separation of ethanol–water mixtures. The membranes were characterized by FTIR to verify the formation of the polyion complex, XRD to observe the effects of blending on crystallinity, DSC, and TGA to investigate the thermal stability, and tensile testing to assess their mechanical stability. The effect of experimental parameters such as feed composition, membrane thickness and permeate pressure on separation performance of the crosslinked membranes was determined. Sorption studies were carried out to evaluate the extent of interaction and degree of swelling of the blend membranes, in pure as well as mixtures of the two liquids. Crosslinked blend membranes were found to have good potential for breaking the azeotrope of 0.135 mol fraction of water and a high selectivity of 436 was observed at a reasonable flux of 0.22 g/(m^2 10 μm h). Membrane selectivities were found to improve with decreasing membrane pressure but remained relatively constant for variable membrane thickness. Increasing membrane thickness decreased the flux and higher permeate pressure caused a reduction in both flux and selectivity.

Chitosan is one of the most widely used pervaporation membranes in the world today. A novel method for cross-linking membranes using phosphoric acid in alcohol baths was investigated in this study for the separation of ethylene glycol (EG)/water mixtures. The cross-linked membranes were subjected to sorption studies to evaluate the extent of interaction and degree of swelling in pure as well as binary mixtures of the two liquids. In order to gain a more detailed picture of the molecular transport phenomenon, we have performed sorption gravimetric experiments at 30 °C to compute diffusion, swelling, sorption, and permeability coefficients of phosphorylated chitosan (P-CS) membranes in the presence of ethylene glycol and water. The effects of experimental parameters such as feed composition, membrane thickness, and permeate pressure on separation performance were determined. The membranes were characterized before and after cross-linking by FTIR analysis and TGA to verify cross-linking and to observe the thermal degradation range of the polymer. The membrane appears to have a good potential for breaking the boiling mixture of ethylene glycol/water since a moderately good selectivity of 234 was obtained at a reasonable flux of 0.37 kg/(m^2 h). The separation factor was found to improve with decreasing feed water concentration whereas flux decreased correspondingly. Increasing the membrane thickness decreased the flux but had a less profound effect on the separation factor. Higher permeate pressure caused a reduction in flux and an increase in selectivity (Rao et al. 2007).

Blend membranes of chitosan, with PVA, were prepared by solution casting and crosslinked with a urea formaldehyde/sulfuric acid (UFS) mixture. Chitosan was used as the base component in the blend system, whereas PVA concentration was varied from 20 to 60 wt %. Blend compatibility was studied by DSC and FTIR was used to study membrane crosslinking. Membranes were tested for pervaporation dehydration of isopropanol and tetrahydrofuran (THF) at 30 °C in close proximity to their azeotropic compositions. Membrane performance was assessed by calculat-

ing flux and selectivity. Swelling experiments performed in water + organic mixtures at 30 °C were used to explain the pervaporation results. The blend membrane containing 20 wt % PVA when tested for 5 and 10 wt % water–containing THF and isopropanol feeds exhibited selectivity of 4203 and 17,991, respectively. Flux increased with increasing concentration of water in the feed. Selectivity was highest for the 20 wt % PVA-containing blend membrane. The results of this study are unique in the sense that the crosslinking agent used–the UFS mixture-was novel (Rao et al. 2007).

The pervaporation transport process of H_2O-EtOH solution was studied on a chitosan membrane and on a H_2SO_4 crosslinked chitosan membrane. The influence of concentration, temperature, and crosslinking was also studied. The dependence of permeation fluxes on feed concentration showed strong coupling effects existed in the permeation process. That the thermodynamic swelling distribution relationship changed with the feed concentration also showed that a strong coupling effect existed in the thermodynamic swelling process. The permeation fluxes and thermodynamic swelling processes showed analogous relationships versus the concentration in the feed. The high swelling ratio and the high selectivity of the membrane in the thermodynamic swelling distribution process was the basis of high flux and high permselectivity of pervaporation. With an increase of temperature, the permeation fluxes increased quickly, but the swelling ratio of water and EtOH in the membrane scarcely changed. This showed that an increase of temperature promoted the diffusion process but had little influence on permselectivity. The permselectivity of pervaporation depended strongly on the thermodynamic swelling process.

Novel polymer–clay-based composite membranes were prepared by incorporating sodium montmorillonite (Na^+-MMT) clay into quaternized chitosan. The resulting membranes were characterized by FTIR, wide-angle X-ray diffraction (WXAD), and TGA. The effect of membrane swelling was studied by varying the water concentration in the feed. The membranes were employed for the pervaporation dehydration of isopropanol in terms of feed composition and Na^+-MMT clay loading. The experimental results demonstrated that membrane containing 10 mass% of Na^+-MMT clay showed the highest separation selectivity of 14,992 with a flux of 14.23×10^{-2} kg/(m^2 h) at 30 C for 10 mass % of water in the feed. The total flux and flux of water are found to be overlapping each other particularly for clay-incorporated membranes, signifying that the composite membranes developed in the present study involving quaternized chitosan and Na^+-MMT clay are highly selective toward water. From the temperature-dependent diffusion and permeation values, the Arrhenius activation parameters were estimated. The resulting activation energy values obtained for water permeation (Epw) are much lower than those of isopropanol permeation (EpIPA), suggesting that the developed composite membranes have higher separation efficiency for the water–isopropanol system. The estimated Ep and ED values ranged between 8.97 and 11.89, and 7.56 and 9.88 kJ/mol, respectively. The positive heat of sorption (ΔHs) values were obtained for all the membranes, suggesting that Henry's mode of sorption is predominant in the process.

Kariduraganavar et al. prepared chitosan-cellulose composite membranes by cross-linking reaction with 3-methylglutaric anhydride. The cross-linked membranes with chitosan-cellulose were obtained at different chitosan contents in variations from 50 to 100 wt %, and these membranes were applied in the dehydration of ethanol-water mixtures. In particular, it was observed that a composite membrane containing 80% chitosan showed a performance with a separation factor of α =17.1 and a total permeation flux of/ = 326 g/(m^2 h). It was observed that the total permeation flux decreased when the cross-linking increased and the increase in the ethanol content in the feed solution showed an increase in the separation factor. The composite membrane containing 80% chitosan showed excellent performance with good mechanical strength and dehydration performance in the ethanol-water mixture separation.

Chitosan was grafted with polyaniline through oxidative-radical copolymerization using ammonium persulfate as an initiator. The graft material was used to prepare a series of membranes by the variation of aniline ratio. These membranes were characterized by FTIR, WAXD, DSC and SEM. The resulting membranes were tested for their ability to separate water–isopropanol mixtures by pervaporation in the temperature range of 30–50 °C. The membrane containing 1:3 grafting ratio exhibited the highest separation selectivity of 2092 with a flux of 1.19×10^{-2} kg/(m^2 h) at 30 °C for 10 mass % of water in the feed. The total flux and the flux of water are close to each other particularly for the grafted membranes, signifying that these could be used to break the azeotropic point of water–isopropanol mixtures. From the temperature dependency of diffusion and permeation values, the Arrhenius activation parameters were estimated and discussed in terms of membranes efficiency. All the membranes exhibited positive heat of sorption (ΔH_s), giving endothermic contribution.

Using a sol−gel technique, organic−inorganic hybrid membranes were prepared using chitosan and mixed silica precursors such as tetraethoxysilane and γ-glycido xypropyltrimethoxysilane. The γ-glycidoxypropyltrimethoxysilane acted as a coupling agent to enhance the compatibility between the organic (chitosan) and the inorganic (tetraethoxysilane) phase. Different techniques such as FTIR, WAXD, SEM, and TGA were employed to study the physicochemical changes in the resulting membranes. These membranes were tested for their ability to separate water + isopropanol mixtures by pervaporation in the temperature range of (303 to 323) K. The experimental data demonstrated that both flux and selectivity were increased simultaneously with increasing the amount of γ-glycidoxypropyltrimethoxysilane. However, this trend no longer remained when the content of γ-glycidoxypropyltrim ethoxysilane was increased beyond 0.25 mass fraction. The membrane containing 0.25 mass fraction of γ-glycidoxypropyltrimethoxysilane (M-2) exhibited the highest separation selectivity of 18,981 with a thickness-normalized flux of $7.45 \cdot 10^{-7}$ kg/(m h) at 303 K. The total flux and flux of water were found to be overlapping with up to 0.25 mass fraction of γ-glycidoxypropyltrimethoxysilane, suggesting that these membranes could be used effectively to break the azeotropic point of water−isopropanol mixtures. From the temperature-dependent permeation values, the Arrhenius activation parameters were estimated. The activation energy values

obtained for water permeation (E_{pw}) are significantly lower than those of isopropanol permeation (E_{pIPA}), suggesting that the developed membranes have demonstrated an excellent separation performance for water−isopropanol systems.

Dense membranes of chitosan were prepared and ionically crosslinked with phosphoric acid for varying intervals of time. The membranes were characterized by FTIR and XRD to confirm cross-linking. TGA and IEC studies were conducted to assess the thermal stability and estimate the number of interactive groups left in the membrane after crosslinking. Sorption studies were carried out to evaluate the extent of interaction and degree of swelling of the membranes in pure liquids as well as binary mixtures. The phosphorylated chitosan membrane crosslinked for 2 h showed good mechanical strength and strong potential for breaking the azeotrope of 95.58 wt % ethanol by exhibiting a high pervaporation selectivity of 213 with substantial water flux of 0.58 kg/(m^2 h). Pervaporation experimental parameters such as feed composition, membrane thickness and permeate pressure were varied to identify optimum operating conditions.

Using a solution technique, chitosan-wrapped multiwalled carbon nanotubes (Chitosan-wrapped MWCNTs) incorporated sodium alginate (SA) membranes were prepared. After studying the physico-chemical properties of these membranes using FTIR, WAXD, DSC, TGA and SEM, the membranes were subjected to pervaporation dehydration of isopropanol. The effects of Chitosan-wrapped MWCNTs and feed composition on the pervaporation performance of the membranes were systematically studied. The membrane containing 2 mass% of Chitosan-wrapped MWCNTs exhibited the highest separation selectivity of 6419 with a flux of 21.76×10^{-2} (g/m^2 h) at 30 °C for 10 mass% of water in the feed. The total flux and flux of water are found to be almost overlapping each other, manifesting that the developed membranes could be used effectively to break the azeotropic point of water–isopropanol mixtures. From the temperature dependent diffusion and permeation values, the Arrhenius activation parameters were estimated. The activation energy values obtained for water permeation (E_{pw}) are significantly lower than those of isopropanol permeation (E_{pIPA}), suggesting that the developed membranes have higher separation ability for water–isopropanol system. The estimated E_p and E_D values were ranged between 14.42 and 10.79, and 14.97 and 11.73 kJ/mol, respectively. The negative heat of sorption (ΔH_s) values was obtained for all the membranes, suggesting that Langmuir's mode of sorption is predominant.

Novel polyelectrolyte complexes containing free sulfate (SO_3) groups (PECSs) were synthesized, with the sulfation of NH_2 groups in the soluble chitosan (CS)/sodium carboxymethyl cellulose (CMC) complexes, and their membranes (PECSMs) were subjected to pervaporation dehydration of ethanol. Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy were employed to characterize the chemical structure and the composition of PECSs. Zeta (ξ) potential and field emission scanning electron microscopy were used to investigate the surface charge density of the PECS particles and the morphology of their membranes. The effects of the chemical composition on the swelling degree, the hydro-

philic property, and the pervaporation dehydration performance of PECSMs were determined. It was found that free SO_3 groups were successfully incorporated into PECSMs. Both the flux and the separation factor of PECSMs increased with increasing SO_3 groups. The permeation flux of PECSMs increased, and their selectivity was almost invariable with increasing temperature. A high separation performance of PECSMs was achieved in the dehydration of 10 wt % water–ethanol mixtures at 70 °C, yielding a flux and a separation factor for the PECSM-20 at 1385 g/(m^2 h) and 1571, respectively. These results indicated that the introduction of free SO_3 groups into PECSMs was an effective strategy to improve the pervaporation dehydration performance of PECSMs.

Surface-modification of polybenzoimidazole (PBI) membrane with chitosan chains has been performed using 4-isocyanato-4′-(3,3′-dimethyl-2,4-dioxo-azetidino) diphenylmethane (IDD) as a coupling agent to build up chemical linkages between the PBI membrane surface and chitosan chains. Incorporation of chitosan chains to PBI membrane surface increases its surface hydrophilicity and enhances its performance of pervaporation dehydration on isopropanol aqueous solutions. The surface chemical structure of the chitosan-modified PBI membrane (PBI-CS) has been characterized with attenuated total reflectance Fourier transform infrared and X-ray photoelectron spectroscopies. The scanning electron micrographs of PBI-CS indicate the surface reaction between PBI and IDD might take place at the top surface of the PBI matrix in a thickness of about 2 μm as the PBI membrane is swollen with the used solvent. PBI-CS is effective for pervaporation dehydration on isopropanol aqueous solutions in a wide concentration range from 30 to 90 wt%. The chitosan layer increases the dissolution rate of water into the PBI-CS membrane so as to simultaneously increase the water permeability and selectivity of the membrane. PBI-CS shows high pervaporation separation indexes which are about 3.9-fold of the value measured with the neat PBI membrane.

The permeation and separation characteristics of an ethanol-water azeotrope through chitosan membranes of different molecular weights and degrees of deacetylation during pervaporation were investigated (Uragami et al. 2015). The normalized permeation rate decreased with increasing molecular weight up to 90 kDa, but the rate increased at over 90 kDa. On the other hand, the water/ethanol selectivity increased with increasing molecular weight up to 90 kDa but decreased at over 90 kDa. With increasing degree of deacetylation, the water/ethanol permselectivity increased significantly, but the normalized permeation rate decreased. The characteristics of the chitosan membranes were discussed based on their chemical and physical structures, such as the contact angle, density, degree of swelling and glass transition temperature.

Glycidyltrimethylammonium chloride (GTMAC) grafted chitosan (CS) membranes were prepared by the solution casting technique. The chemical composition and morphological characteristics of the prepared GTMAC/CS membranes were investigated by FTIR, WAXD, DSC, TGA and SEM. The effects of grafting and feed composition on pervaporation performance of the membranes were systematically studied. The membrane containing 40 mass % of GTMAC exhibited the

highest separation selectivity of 2133 with a flux of 6.91×10^{-2} kg/(m^2 h) at 30 °C for 10 mass % of water in the feed. The total flux and flux of water are almost overlapping each other, manifesting that these membranes could be used effectively to break the azeotropic point of water–isopropanol mixture. From the temperature dependent diffusion and permeation values, the Arrhenius activation parameters were estimated. The activation energy values obtained for water permeation (E_{pw}) are significantly lower than those of isopropanol permeation (E_{pIPA}), suggesting that the grafted membranes developed here have higher separation ability for water-isopropanol system. The positive heat of sorption (ΔH_s) values was obtained for all the grafted membranes, suggesting that the Henry's mode of sorption is predominant.

The blocked diisocyanate crosslinked chitosan membrane was modified by incorporating different mass % of NaY zeolite. The physico-chemical properties of resulting composite membranes were studied using FTIR, WAXD, TGA, DSC and SEM. The mechanical properties of the membranes were studied using universal testing machine. After measuring the equilibrium swelling, membranes were subjected to pervaporation for separation of water–isopropanol mixtures. Both flux and selectivity were increased with increasing NaY zeolite content in the membranes. The membrane containing 40 mass % of NaY zeolite exhibited the highest separation selectivity of 11,241 with a flux of 11.37×10^{-2} kg(m^2 h) or 10 mass % of water in the feed. The total flux and flux of water are almost overlapping each other, suggesting that these membranes could be effectively used to break the azeotropic point of water–isopropanol mixture. From the temperature dependent diffusion and permeation values, the Arrhenius activation parameters were estimated. All the composite membranes exhibited lower activation energy compared to crosslinked membrane, indicating that the permeants require less energy during the process because of molecular sieving action attributed to the presence of sodalite and super cages in the framework of NaY zeolite. The Henry's mode of sorption dominates the process, giving an endothermic contribution.

Polyelectrolyte complexes with sulfonate ionic cross-linking and free sulfate groups were fabricated from chitosan and poly (sodium vinylsulfonate) (PVS) *via* the "complexation-sulfation" method. Homogenous membranes (S-PVS/CS PECMs) were prepared exhibiting both high flux and water selectivity in dehydrating various alcohols and the separation performance was proportional to the content of free sulfate groups. For example, in dehydrating 10 wt % water/ethanol mixture at 70 °C, the flux and water content in permeate for the S-PVS/CS-3 PECM was 1980 g/(m^2 h) and 99.55 wt %, respectively. Moreover, the separation performance was stable versus an operation time of 120 h and could dehydrate an azeotropic water/ethanol mixture to fuel grade ethanol. Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy, and ζ potential were employed to characterize chemical structures, compositions, and charge properties of S-PVS/CS PECs. Scanning electron microscopy and contact angle measurement were exploited to investigate the morphology and hydrophilicity of S-PVS/CS PECMs. In addition,

swelling and sorption behavior of S-PVS/CS PECMs were determined to interpret separation performances (Zheng et al. 2016).

Box–Behnken (BB) design of response surface methodology (RSM) was effectively applied to optimize fabrication conditions of modified PVA and chitosan blende PV membranes. The PVA/chitosan membranes were crosslinked either by chemical reaction with glutaraldehyde or by heat-treating at different temperatures. The main objectives were to determine the optimal levels of fabricating parameters and also to investigate interactions among the variables. Chitosan in the blended membranes, concentration of crosslinking agent and heat-treating temperature were the fabrication parameters, the main effects and interaction effects of which on membrane structure and PV performance toward isopropanol (IPA)/water dehydration were investigated, and for which regression models were established. The modified PVA/chitosan blend membranes were characterized by means of SEM, FTIR as well as XRD. It was found that the chitosan content is the most significant factor influencing flux and separation factor among the three studied variables and the experimental results are in excellent accordance with predicted values from the developed RSM regression models. The RSM results indicated that under preparation conditions of 80 wt % chitosan blended membrane, 0.58 wt % GA concentration, and 77 °C heat-treating temperature, the maximum separation factor of 5222.8 and the normalized flux of 9.407 kg μm/(m^2 h) can be acquired with feed content of 85 wt % IPA at 25 °C, showing that the prepared membrane is highly efficient for PV dehydration of IPA. The models were satisfactorily validated against experimental data. Furthermore, the optimum membrane presents excellent separation performance at different feed compositions and temperatures.

The blended membranes of PVA and chitosan were prepared for pervaporation dehydration of a binary system of *n*-butyl acetate/water and a ternary mixture of *n*-butyl acetate/*n*-butanol/water. Membranes were characterized by FTIR, DSC and XRD to investigate the intermolecular interactions, the blending compatibility and the effects of blending on crystallinity. Tensile strengths were measured to determine the influence of blending ratios on the membrane's mechanical properties. The membrane's degree of swelling in water dropped significantly with increasing chitosan. The results agreed well with the calculation of the solubility parameter. Separation factor and permeation flux increased when the temperature was raised for both the binary and ternary pervaporation systems. An optimal separation factor of 27,000 with a total flux of 402 g/(m^2·h) was obtained using the blend membrane containing 25 wt % chitosan for the binary system. For the ternary system, the maximum separation factor and total flux were obtained for blend membranes at 75 wt % and 50 wt % chitosan content, respectively.

A new two-dimensional microporous metal organic framework (MOF), Al-MOF, [Al(OH)(MBA)] (CYCU-7, MBA = diphenylmethane-4,4′-dicarboxylate anion) and its reported analogue, [Al(OH)(SBA)] (CAU-11, SBA = 4,4′-sulfonyldibenzoate anion), have been synthesized using hydrothermal and solvothermal methods, respectively, and their structural crystallinities and defect porosities were carefully controlled and characterized by N_2 sorption isotherms and ^{27}Al solid-state nuclear magnetic resonance measurements. Interestingly, the MOF synthesized by the ethanol-based solvothermal method (CYCU-7) show a significant degree of

linker-missing defects compared to that synthesized by the water-based hydrothermal method (CAU-11). We further incorporated the synthesized CYCU-7 and CAU-11 with chitosan (CS) biopolymer to make CYCU-7@CS and CAU-11@CS mixed matrix membranes (MMMs) with the loading amount of MOF 2.5, 5.0, or 10 wt %. The as-prepared CYCU-7@CS and CAU-11@CS MMMs were applied for separation of water/ethanol mixtures through the pervaporation process, and the effects of the structural properties (*e.g.* crystallinity and defects) of CYCU-7 and CAU-11 on the separation performance are studied. It is found that defect-rich CYCU-7@CS MMMs exhibit higher flux, while CAU-11@CS MMMs with higher crystallinity exhibit a higher separation factor. In addition, the CAU-11@CS MMM with 5.0 wt % loading of CAU-11 displays the best separation performance (separation factor = 2741 and flux = 458 g/m^2 h).

5.3.6.2 Organic-Permselective Membranes

The pervaporation behaviors of aqueous ethanol mixtures through the poly(ethylene oxide) (PEO)/CS blend membrane were investigated. The results show that both CS and PEO/CS membrane preferentially permeate ethanol at a lower alcohol concentration in feed, and the selectivity of CS membrane toward alcohol can be greatly improved by introducing hydrophilic polymer PEO into CS. The PEO/CS blend membrane gave a separation factor of 4.4 and a flux of 0.9 kg/(m^2 h) for 8 wt % of ethanol in the feed at 20 °C. At the same time, the reason introducing PEO can improve alcohol-permselectivity of CS membrane is explained on the basis of experimental data. Blending with PEO made the structure of CS chain looser, which resulted in ethanol molecules passing through easily, on the other hand, strengthened the ability of forming water clusters that inhibit the permeation of water molecules. From the experimental results, although the PEO/CS blend membrane was not a usable membrane with high selectivity to alcohol, a new method to prepare alcohol-permselective membranes appears to be developed by modifying hydrophilic polymers.

5.3.6.3 Organic-Organic Separation Membranes

A benzoylchitosan (B-Cs) membrane material for separation of benzene/cyclohexane (Bz/Chx) was synthesized by Uragami et al. BzCses of varying degrees of benzoylation were synthesized as membrane materials having good durability for the separation of Bz/Chx mixtures. BzCs membranes showed high benzene permselectivity for Bz/Chx mixtures of 50 wt % benzene in PV; the difference in benzene perm- selectivity for BzCs membranes with differing degrees of benzoylation corresponded to a difference in membrane physical structures, based on the characteristics of these membranes. When a Bz/Chx mixture of 50 wt % benzene was permeated through the BzCs membranes, permeation rates increased and benzene permselectivity slightly decreased with increasing degree of benzoylation, as shown in Fig. 5.15.

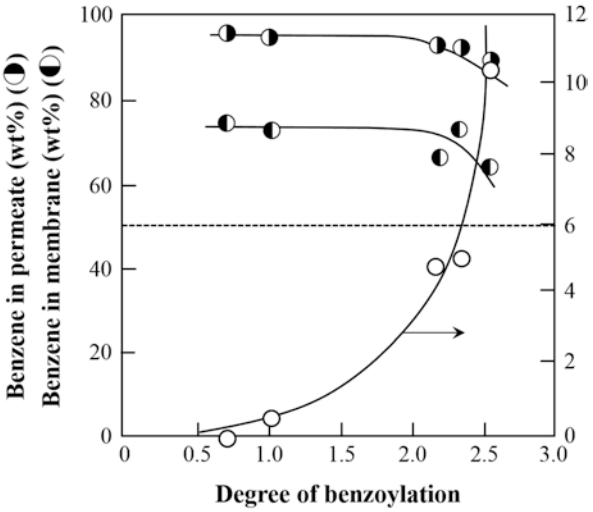


Fig. 5.15 Effects of the degree of benzoylation on the benzene concentration in the permeate (◐) and permeation rate (○) through the benzoylchitosan membranes for benzene/cyclohexane sorbed into their membranes (◑) for benzene/cyclohexane mixture. The dotted line is the feed mixture composition (benzene/cyclohexane = 50/50, w/w)

BzCses with a different degree of benzoylation were synthesized as membrane materials having a good durability for the separation of Bz/Chx mixtures. Characteristics of BzCs membranes such as contact angle, crystallinity and degree of swelling were significantly influenced by the degree of benzoylation. The BzCs membranes showed a high benzene-permselectivity for a Bz/Chx mixture of 50 wt % benzene in pervaporation and a difference of the benzene-permselectivity for the BzCs membranes with different degree of benzoylation corresponded to a difference in the physical structure of the membranes based on the characteristics of these membranes. When a Bz/Chx mixture of 50 wt% benzene was permeated through the BzCs membranes, permeation rate increased and benzene-permselectivity slightly decreased with increasing degree of benzoylation. These results are discussed from the viewpoints of chemical and physical structures of the BzCs membranes with different degree of benzoylation.

Nam and Lee (1999) investigated the efficiency of pervaporation separation of methanol/methyl-*t*-butyl ether mixture through chitosan composite membrane modified with sulfuric acid and four surfactants Effects of feed concentration, temperature, crosslinking degree and type of surfactants were studied. The chitosan composite membrane modified with sulfuric acid showed the pervaporation performance of over 70 wt % methanol in the permeate and flux of 100 g/(m^2 h) measured at 25 °C. At 50 °C, the separation factor decreased while the flux increased exceeding 300 (g/m^2 h). For the membrane complexed with surfactants, the permeate showed 98.3 wt% methanol concentration and 470 g/(m^2 h) of permeate flux at 25 °C. With increasing operating temperature, the permeate flux remarkably increased to 1170 g/(m^2 h) and the permeate showed 97.8 wt % methanol concentrations.

Clear blends of chitosan with poly(*N*-vinyl-2-pyrrolidone) (PVP) made from aqueous solutions appear to be miscible from visual appearance. Infrared (IR)

spectra used to investigate the carbonyl-hydroxyl hydrogen bonding in the blends indicated compatibility of two polymers on a molecular level. The IR spectra were also used to determine the interaction change accessing with increasing temperature and indicated that a significant conformational change occurred. On the other hand, the blend membranes were evaluated for separation of methanol from methyl tert-butyl ether. The influences of the membrane and the feed compositions were investigated. Methanol preferentially permeates through all the tested membranes, and the partial flux of methanol significantly increase with the poly(*N*-vinyl-2-pyrrolidone) content increasing. The temperature dependence of pervaporation performance indicated that a significant conformational change occurred with increasing temperature. Combined with the IR results, the pervaporation properties are in agreement with characteristics of interaction between chain–chain within the blend membranes.

The polyion complex composite (PIC) membranes were prepared by the complexation of the ionic groups of sodium alginate and chitosan, and the pervaporation characteristics were investigated for the separation of MTBE/methanol mixtures. The polyion complex reaction took place by the ionic crosslinking reaction between the carboxylate groups ($–COO^-$) of sodium alginate and the protonated amine groups ($–NH_3^+$) of chitosan. The polyion complexation depended upon the content of counter ions. In this study, the prepared membranes showed excellent pervaporation performance in the separation of MTBE/methanol mixtures. Especially, PIC membrane prepared from 2.0 wt % SA solutions and 2.0 wt % chitosan solution appeared to permeate only methanol from the feed, with the flux of over 240 g/(m^2 h). As the operating temperature increased from 40 to 55 °C, the permeation rate of methanol increased, but that of MTBE decreased. These results were due to the physicochemical and structural properties of polyion complex membranes.

Composite chitin membranes supported by porous polyetherimide substrate were prepared for the pervaporation separation of ethanol/toluene and methanol/toluene mixtures. The chitin was obtained by modifying chitosan to its original form chitin by the *N*-acetylation reaction. It was found that the incorporation of additional acetyl groups into the chitosan structure decreased the total flux and increased separation factor from 401 g/m^2 h; α = 34 (pure chitosan) to 282 g/(m^2 h); α = 126 (4 mol acetylated chitosan) for 10% EtOH feed mixture and from 681 g/(m^2 h); α = 159 (pure chitosan) to 484 g/(m^2 h); α = 607 for 10% MeOH feed mixture. It was concluded that chitin composite membranes could be a good candidate for this pervaporation system. SEM and FTIR determinations of the acetylated chitosan membranes were carried out and are reported. It was further shown that the chitin/polyetherimide composite membranes prepared had good pervaporation characteristics and were also found to be mechanically robust and stable to withstand the corrosive nature of the ethanol/toluene mixture during the pervaporation runs. This is the first reported successful application of chitin in the form of composite membranes for the pervaporation separation of organic/organic liquid systems (Huang et al. 2000).

The separation of binary dimethyl carbonate (DMC)/methanol, DMC/water, and methanol/water mixtures as well as ternary DMC/methanol/water mixtures by pervaporation using chitosan membranes was investigated by Lawless et al. It is relevant to the manufacturing of DMC, where the energy intensive extractive distillation or pressure swing distillation is used conventionally for the separation of the reaction mixtures. Chitosan membranes were prepared by solution casting, followed by alkaline treatment. The effects of feed composition and operating temperature on the separation performance were investigated, and the membrane properties under the experimental conditions that are of interest to the manufacturing of DMC were evaluated. It was demonstrated that the membrane exhibited good performance for the DMC/methanol separation as well as the dehydration of DMC. The membrane also showed the capability of dehydrating methanol, but with a lower permselectivity. For the separation of ternary DMC/methanol/water mixtures, the interactions among the permeating components were shown to have a significant effect on the membrane performance.

For the separation of methanol/MTBE (methyl *tert*-butyl ether) mixtures, methanol selective chitosan composite membranes were prepared and tested for pervaporation experiments. When anionic surfactants are added into the cationic chitosan solution, the solution viscosity was drastically decreased due to the collapsed chain conformation. Pervaporation characteristics of surfactant modified chitosan membrane were substantially improved due to the decreased membrane thickness and possible enhanced affinity to methanol. Rheological data of the casting solution was measured using viscometer and the surface morphology of the surfactant complexed chitosan membrane was investigated by AFM.

Poly(vinyl alcohol)/chitosan (PVA/CS) blend membranes were prepared by mixing the two polymeric homogeneous solutions. The interaction between PVA and chitosan arising from hydrogen bond was analyzed through intrinsic viscosity determination and FTIR spectra, and there existed an incompatibility region in the blend membranes when chitosan content was 50 wt %. XRD showed that the crystalline structure of PVA and chitosan was disrupted through blending. The incompatibility of two polymers and disrupted crystalline structure led to a less compact structure. The blend membranes were used to pervaporative separation of benzene/cyclohexane (Bz/Chx) mixtures. The effects of chitosan content in blend membranes, weight fraction of benzene in feed, and operating temperature on separation performance of Bz/Chx mixtures were investigated. Compared with pure PVA and pure chitosan membranes, blend membranes showed much higher permeation flux and slightly higher benzene permselectivity. As the weight fraction of benzene in feed increased, separation factor decreased but total permeation flux increased. And it was observed that total permeation flux increased and separation factor decreased with increasing operating temperature. The total permeation flux of blend membrane with 50 wt % chitosan was 51.41 g/(m^2 h) and separation factor was 49.9 for Bz/Chx mixtures with 50 wt % of benzene at 323 K (Lu et al. 2006).

Acetone in view of lower toxicity could be used as extracting solvent of lycopene in tomato paste instead of hexane. The studied chitosan membranes were uncrosslinked and crosslinked types. It was found that the membrane morphology could be

changed from porous to dense structure by increasing evaporation temperature or time or by crosslinking. They showed hydrophilicity but not preferred to both acetone and lycopene. The lycopene/acetone mixture could be separated by using water swollen chitosan membrane in pervaporation process. It was found that all studied membranes; i.e., both uncrosslinked and ionically crosslinked membranes prepared at 40–60 °C for 2–6 h, could separate acetone from lycopene solution as permeate with rejection of 100% or separation factor of infinity. The uncrosslinked membrane prepared at 40 °C for 2 h provided the highest acetone flux of 0.131 ± 0.004 L/(m^2 h) by operating at −1 bar and 15 ppm feed concentration.

Chitosan (CS)/polyvinylpyrrolidone (PVP)-silica hybrid membranes are prepared to separate the methanol/ethylene glycol (EG) azeotrope. These hybrid membranes are formed in semi-interpenetrating network structure at the molecular scale via sol-gel reactions between CS and tetraethoxysilane. The physico-chemical property and morphology of the as-prepared membranes are investigated in detail. They have lower crystallinity, higher thermal stability, and denser structure than the pristine CS membrane and its blending counterpart. The as-prepared hybrid membranes demonstrate excellent performances and a great potential in pervaporation separation of methanol/EG. Silica-hybridization depressed the swelling degree of membranes in the azeotrope, and remarkably enhanced methanol sorption selectivity. The membrane containing 7.77 wt % PVP and 14.52 wt % TEOS has a permeation flux of 0.119 kg/(m^2 h) and separation factor of 1899.

Functionalized multiwalled carbon nanotubes (MWNTs) were incorporated into a chitosan membrane for separation of benzene/cyclohexane mixtures by pervaporation. The pristine MWNTs were treated by mixed acid (H_2SO_4/HNO_3 = 3/1) and then functionalized with isonicotinic acid. Ag^+ was grafted to the pyridine ring on the MWNTs by a complexation reaction. Membranes made of chitosan and Ag^+/carbon nanotubes were prepared with polysulfone membranes as support. The surface structure of modified MWNTs was examined by FT-IR, TEM, EDS and XPS to assess its morphology. The swelling and pervaporation characteristics of the membranes for separation of benzene/cyclohexane mixtures were investigated. The swelling degree of the membranes in benzene was more than 2.5 times that of the pristine chitosan membrane. The swelling degree was found to increase with the content of the MWNTs–Ag^+ in the membrane and the benzene content in the feed. Pervaporation results also showed that the MWNTs–Ag^+/chitosan hybrid membrane separation performance is better than MWNTs/chitosan hybrid membrane and pristine chitosan membrane. The permeation flux of the membrane increased, the selectivity increased firstly and then decreased with the increase in the content of MWNTs–Ag^+. The selectivity reached 7.89 and the permeate flux was 357.96 g/(m^2 h) (the mass ratio of benzene was 50%, the temperature was 20 °C) when the percentage by weight of the MWNTs–Ag^+ to chitosan was 1.5%. The Arrhenius activation parameter of 1.5% MWNTs–Ag^+/chitosan (CS) hybrid membrane is 4.28 kJ/mol while 1.5% MWNTs/CS membrane is 10.6 kJ/mol in 50% benzene concentration in feed.

Mixed matrix membranes (MMMs) of chitosan were prepared by incorporating silicalite zeolite particles in 5 and 10 wt % with respect to weight of chitosan polymer to improve their pervaporation (PV) separation characteristics over that of pristine

chitosan membrane for toluene/methanol and toluene/ethanol feeds in compositions of 10–40 wt % of toluene at 30 °C. The membranes were crosslinked with glutaraldehyde (GA), which was confirmed by FTIR and ion exchange capacity (IEC) measurements. X-RD and SEM studies were performed to understand membrane morphology. Toluene permeated preferentially with a selectivity of 264 and fluxes of 0.019–0.027 kg/(m^2 h) for toluene/methanol mixture. Selectivity of 301 with fluxes ranging from 0.019 to 0.026 kg/(m^2 h) was observed for toluene/ethanol mixtures. Flux increased, while selectivity decreased with increasing toluene content of the feeds. An increase in silicalite content of the MMMs gave increased PV performances.

The synthesis of a novel chitosan-polytetrafluoroethylene composite membrane with solvent resistant property for efficient separation of methanol/toluene mixture by pervaporation. The composite was crossed with tetraethyl orthosilicate (TEOS) to prevent or reduce membrane swelling and improve the separation factor. The synthesized membranes were characterized by SEM, FTIR and DSC analysis. Molecular dynamics (MD) simulation and computational fluid dynamics were coupled to predict the structural and diffusive properties besides concentration profile inside the membrane. Diffusion coefficients of methanol and toluene were found to be 1.7×10^{-9} and 1.8×10^{-12} m^2/s, respectively. The effect of crosslinking on process parameters such as flux and separation factor was analyzed. The study confirmed that increasing TEOS concentration reduced the methanol flux but enhanced separation factor with respect to this alcohol. The membranes exhibited a flux of 0.13 kg/(m^2 h) and separation factor of 58.4 for azeotropic feed composition of 68 wt% methanol.

5.3.7 Technology of Evapomeation

In Table 5.1 the permeation and separation characteristics for various aqueous ethanol solutions through the chitosan membrane by EV and PV are compared (Uragami et al. 1988).

The permeation rates for both EV and PV decreased with increasing ethanol concentration in the feed. These results suggest that the chitosan membrane becomes dense as the ethanol content in the feed increases. The permeation rates for EV were smaller by one order of magnitude compared with those for PV. This supports the assumption that the chitosan membrane can almost fully maintain its pre-test dense structure during evapomeation, and that consequently, the diffusivity of the permeating species during the diffusion process is lowered. As can be seen from Table 5.1, water was predominantly permeated through the chitosan membrane both in PV and EV. An azeotropic composition, viz. 95.6 wt % ethanol in the feed solution, was not observed for both methods, and water permselectivities for EV were greater than those for PV. These results depend on the fact that swelling of the CS membrane is much more inhibited during EV than during PV (Uragami 2017).

Table 5.1 Permeation and separation characteristics for aqueous ethanol in pervaporation (PV) and evapomeation (EV)

PV			EV	
Ethanol in feed (wt%)	Permeation rate (10^{-2} kg/m^2 h)	Separation factor ($\alpha_{H2O/EtOH}$)	Permeation rate (10^{-2} kg/m^2 h)	Separation factor ($\alpha_{H2O/EtOH}$)
0	186.0	–	176.0	
10 (43.9)	150.0	0.7	148.0	5 (33)
30 (60.4)	136.0	2	126.0	7 (25)
50 (67.7)	67.1	13	95.6	26 (56)
70 (77.3)	34.6	50	39.2	37 (53)
90 (90.8)	12.3	31	18.0	114 (124)
96.5[α]	6.5	17	7.3	202
100	2.9	–	6.2	

Values in parentheses are for vapor compositions
α: Azeotrope

Permeation rate and separation factor for aqueous dimethyl sulfoxide solutions through a membrane in pervaporation and evapomeation as a function of the dimethyl sulfoxide concentration in the feed solution or feed vapour are shown in Fig. 5.16, in which the separation factor $\alpha_{H20/DMS0}$ is expressed as

$$\alpha_{H_2O/DMSO} = \left(Y_{H_2O} / Y_{DMSO}\right) / \left(X_{H_2O} / X_{DMSO}\right) \tag{5.1}$$

Where X_{H2O}, X_{DMSO} and Y_{H2O}, Y_{DMSO} are the weight fractions of water and dimethyl sulfoxide in the feed solution and permeate respectively in pervaporation or are the weight fractions of water and dimethyl sulfoxide vapours from the feed solution and in the permeate respectively in evapomeation.

In order to confirm the discussion for the dehydration from aqueous dimethyl sulfoxide solution and vapour in pervaporation and evapomeation through a hydrophilic chitosanmembrane, similar experiments were carried out for aqueous acetic acid solution and vapour using hydrophobic poly(vinyi chloride) membrane.

The effect of the feed vapor composition of the aqueous ethanol solutions on the permeation rate, the ethanol concentration in the permeate through the chitosan and glutaraldehyde crosslinked chitosan (GAC) membranes in evapomeation, and the degree of swelling of the membrane are shown in Fig. 5.17 (Uragami et al. 1994).

The GAC membrane contained 0.5% glutaraldehyde (3.2% glutaraldehyde in the casting solution). Both the CS and GAC membranes had a low ethanol concentration in the permeate and showed high water permselectivity. There were also significant differences between the permeate compositions of the CS and GAC membranes: the GAC membrane had a higher permeate com position than the CS membrane despite the fact that the permeation rate of the GAC membrane was greater than that of the CS membrane.

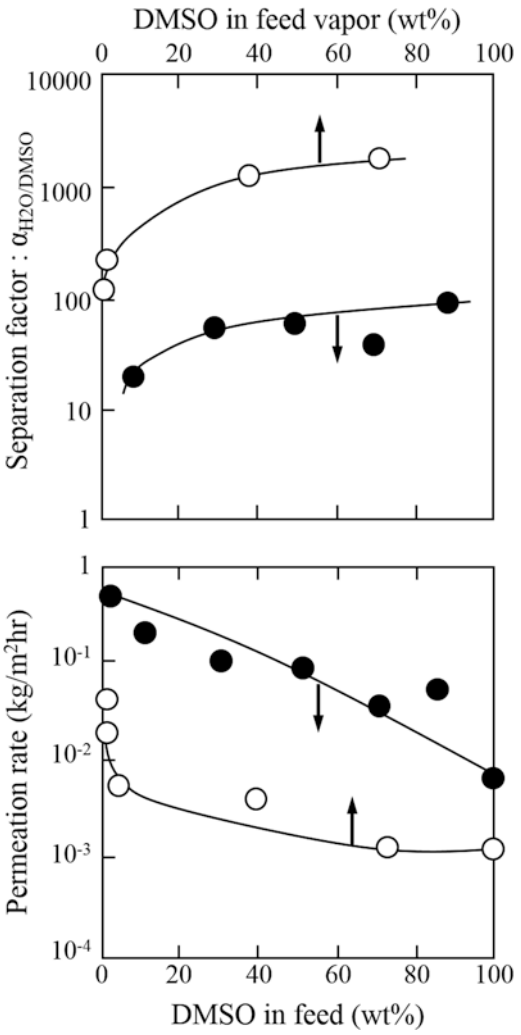


Fig. 5.16 Permeation rate and separation factor for aqueous dimethyl sulfoxide solution through the chitosan membrane in pervaporation(○)and evapomeation(●)and as a function of the dimethyl sulfoxide concentration in the feed solution or feed vapor

Furthermore, the degree of swelling of the GAC membrane was higher than that of the CS membrane, and this tendency increased with decreasing ethanol concentration in the feed mixture. When polymer membranes are cross-linked, permselectivity is generally improved, but the permeation rate decreases because the degree of swelling of the membrane is lowered. In this case, however, the permeation rate, water permselectivity and degree of swelling of the GAC membrane were higher than those of the CS membrane. To clarify the results in 17, the density and crystallinity of the CS and GAC membranes were determined by the flotation method and wide-angle X-ray diffraction, respectively. The density and the correlation crystallinity index decreased with an increasing GA content in the casting solution. These results imply that the increase in the cross-linking of the chitosan

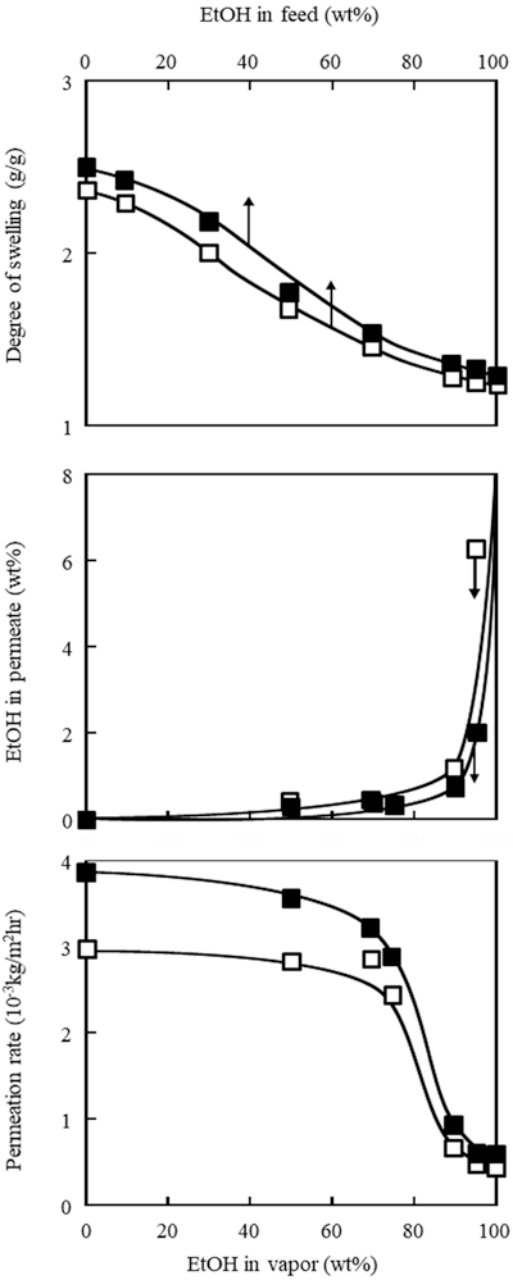
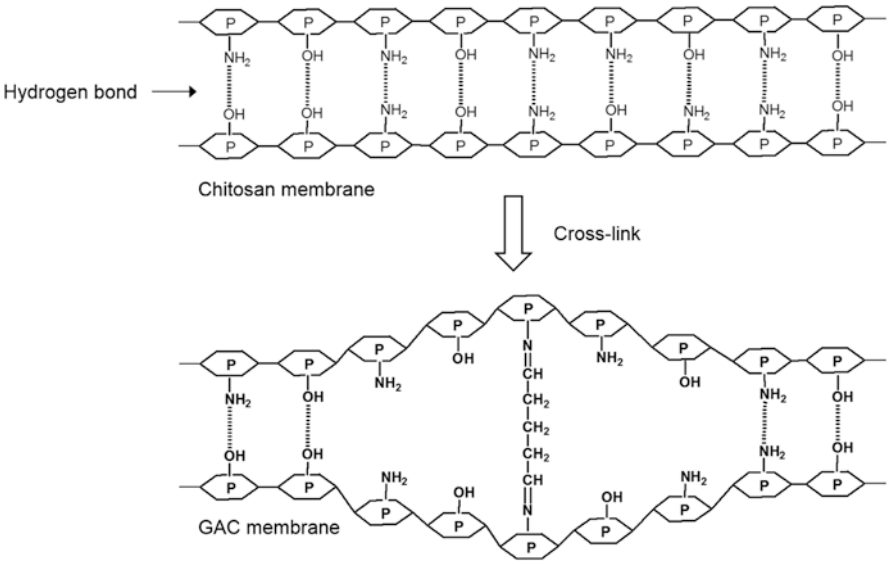


Fig. 5.17 Effects of ethanol concentration in the feed vapor on the permeation and separation characteristics and degree of swelling of the chitosan (□) and GAC (■) membranes by evapomeation



Scheme 5.1 Model structures of the chitosan and GAC membranes. P: pyranose ring unit; ····: hydrogen bond

membrane decreases the density and the crystallinity of the membrane. From these results, a model structure, as shown in Scheme 5.1, is assumed for the chitosan and GAC membranes.

The CS membrane has many intermolecular hydrogen bonds between hydroxyl groups and amino groups. A few of these hydrogen bonds in the GAC membrane are broken by cross-linking with glutaraldehyde, and free hydrophilic groups such as hydroxyl and amino groups are formed. These hydrophilic groups have a strong affinity to water molecules, that is, the solubility of water molecules into the GAC membrane is increased. On the other hand, since the water molecules sorbed into GAC membranes are smaller than ethanol molecules, the water molecules can be more easily diffused in the GAC membrane than ethanol molecules. Consequently, GAC membranes are moderately swollen by water molecules and simultaneously increase the water/ ethanol selectivity. The increase of water/ethanol selectivity in the GAC membrane is due to both the increase in the solubility of water molecules into the GAC membrane and the increase in the diffusivity of water molecules in the GAC membrane. From this discussion, both the increase in the permeation rate and the separation factor with increasing GA content cross-linked in the CS membrane can be understood.

If a deformation of the hydrogen bonds in the CS membrane occurs as shown in Scheme 5.1, high permeation rate and high water permselectivity result; it can be assumed that the cross-links are not always required. Thus chemical modification of the CS membrane was tried using *N*-alkyl aldehyde as a monofunctional aldehyde and these *N*-alkyl CS membranes were applied to the permeation and separation of aqueous ethanol solutions by evapomeation (Uragami et al. 1997) between the

increase in the hydrophilicity of the *N*-alkyl CS membranes based on the deformation of the hydrogen bonds in the CS membrane and the increase in the hydrophobicity of the membranes due to the *N*-alkylation of the amino groups in the CS membrane was appropriate, both permeation rate and water permselectivity were improved by alkylation of the CS membrane.

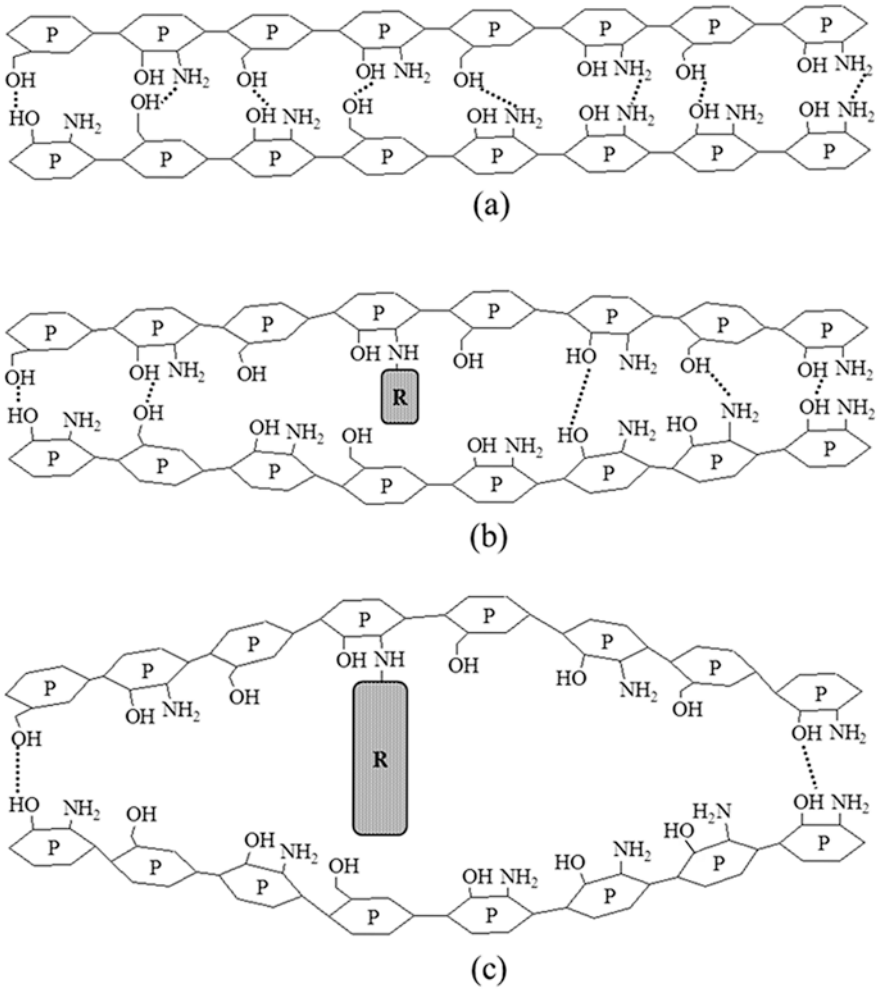
In 18 the effect of number of carbons in the *N*-alkyl group of the chitosan molecule on the permeation rate and the separation factor for an aqueous solution of 10 wt % ethanol through *N*-alkyl chitosan membranes in evapomeation is shown.

The degree of substitution of the *N*-alkyl chitosan membranes used in this section was almost equal at about 3 mol %. In an *N*-ethyl chitosan membrane, the permeation rate and the separation factor were almost equal to those in the chitosan membrane. But both the permeation rate and the separation factor of an *N*-propyl chitosan membrane were smaller than those of the chitosan membrane. In contrast, the permeation rate of an *N*-butyl chitosan membrane was slightly smaller than that of the chitosan membrane but the separation factor of the *N*-butyl chitosan membrane was about 1.5 times that of the chitosan membrane. Furthermore, in an *N*-pentyl chitosan membrane the separation factor was about 2 times greater than that in the chitosan membrane. In addition, both the separation factor and the permeation rate in the *N*-pentyl chitosan membrane showed a maximum.

From the aforementioned results, the model depicted in Scheme 5.2 is proposed to provide an understanding of the relationship between the permeation and separation characteristics for aqueous ethanol solutions through *N*-alkyl chitosan membranes in evapomeation and the changes in their membrane structure. Namely, the chitosan membrane has a very dense structure based on the hydrogen bonds among the hydroxyl groups, amino groups, and hydroxyl and amino groups in the chitosan chains as shown in Scheme 5.2a. However, introducing the *N*-alkyl group severs some of the hydrogen bonds as shown in Scheme 5.2b, c, the physical structure of the *N*-alkyl chitosan membrane becomes rough and the density of the *N*-alkyl chitosan membrane is decreased. Such an effect is remarkable for a chitosan membrane having bulky alkyl groups. On the other hand, the chitosan membrane becomes hydrophobic by alkylation of the hydrophilic amino groups. Therefore, the *N*-alkyl chitosan membrane has a strong affinity for an aqueous solution of 96 wt % ethanol which is a relatively hydrophobic feed mixture, and has a high degree of swelling. Consequently, the permeation rate is increased and the separation factor is decreased with an increase in the number of carbon atoms.

Quaternized chitosan (*q*-Chito) and cross-linked *q*-Chito cross-linked with dietylene glycol diglycidyl ether (DEDGE) as shown in Scheme 5.3 membranes were prepared for the dehydration of an ethanol-water azeotrope. These membranes were highly water permselective (Uragami et al. 2002).

The permeation rates of the *q*-Chito membranes decreased with an increasing degree of quaternization, and the cross-linked *q*-Chito membranes also decreased with an increase in the cross-linker concentration. The decrease in the permeation rate of the *q*-Chito membrane with an increase of the degree of quaternization was due to a lowering of the diffusivity of the ethanol molecule during the diffusion process, based on the introduction of bulky quaternary ammonium groups into the chitosan molecule chains.



Scheme 5.2 Model structures of the chitosan and *N*-alkyl chitosan membrane. R: alkyl group, P: pyranose ring unit,·····: hydrogen bond. (**a**) chitosan membrane (**b**) N-ethyl, N-propyl chitosan memebrane (**c**) N-butyl, N-pentyl chitosan memebrane

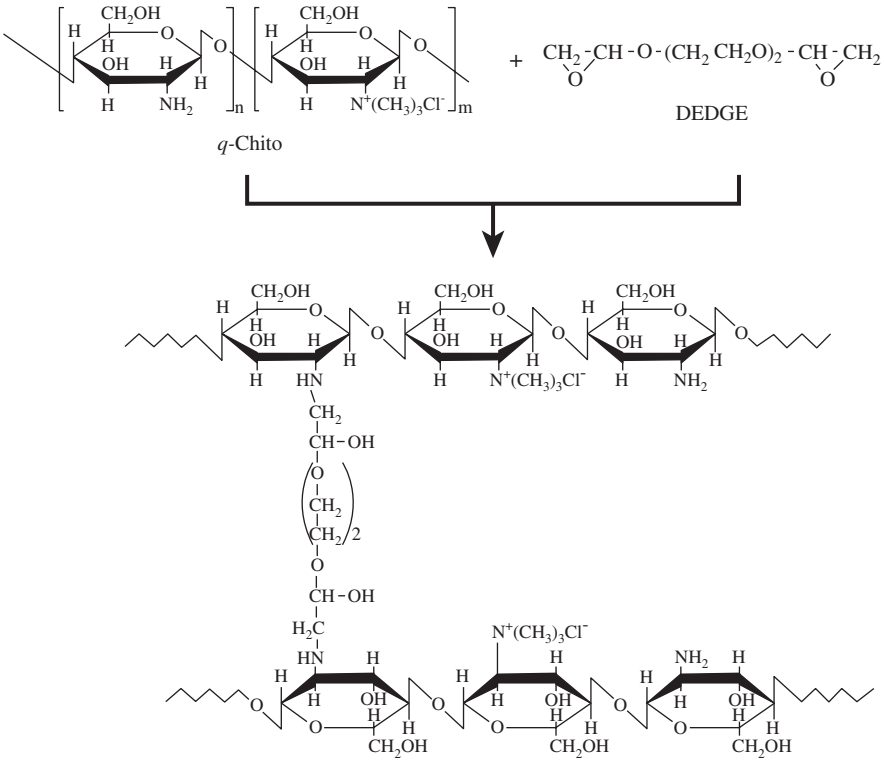
The cross-linking of q-Chito with DEDGE is as follows:

The decreased permeation rate of the amount of cross-linked *q*-Chito membrane with an increase in the cross-linker could be attributed to a lowering of the diffusivity of the permeating molecules caused by the dense cross-link structure between the a-Chito molecule chains. The separation factors for the water permselectivity of the former membrane were mainly dependent on the sorption selectivity based on the solubility of the water molecule into the *q*-Chito membrane. In contrast, the cross-linked *q*-Chito membrane was significantly influenced by both sorption and

diffusion selectivities. The separation factors of the cross-linked *q*-Chito membranes were very high, about 4100–4200. The degree of swelling of these membranes, the membrane density and the contact angle of the membrane surface were consistent with these permeation and separation characteristics for an ethanol-water azeotrope during evapomeation. The separation mechanism for an ethanol-water azeotrope during evapomeation through the *q*-Chito and cross-linked *q*-Chito membranes was analyzed by the solution-diffusion model. The water permselectivity of the *q*-Chito membrane was mainly governed by the sorption selectivity and that in the cross-linked *q*-Chito membrane was dependent on both the sorption and the diffusion selectivity (Fig. 5.18).

PIC cross-linked chitosan membranes (see Scheme 5.3), constructed from *q*-Chito and poly(ethylene oxydiglycolic acid) (PEO acid) on a porous poly(ether sulfone) (PES) (*q*-Chito-PEO acid PIC-PES), as shown in Fig. 5.19, have been applied to the dehydration of an ethanol-water azeotrope in evapomeation (Uragami et al. 2003).

Figure 5.20 shows the permeation rate and the separation factor for the water permselectivity of ethanol-water azeotrope (96.5 wt % ethanol) through *q*-Chito-PEO acid PIC-PES composite membranes as a function of the molar ratio of the



Scheme 5.3 Crosslinking of *q*-Chito with DEDGE

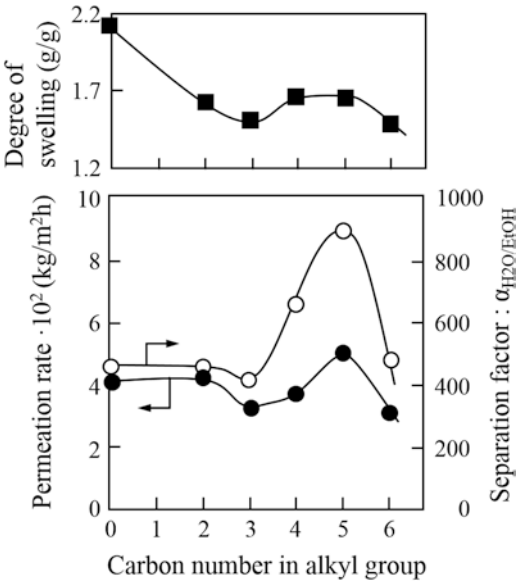


Fig. 5.18 Permeation rate (●), separation factor (○) and degree of swelling (■) of the *N*-alkyl chitosan membranes for an aqueous solution of 10 wt% ethanol in evapomeation as a function of the carbon number of the *N*-alkyl group

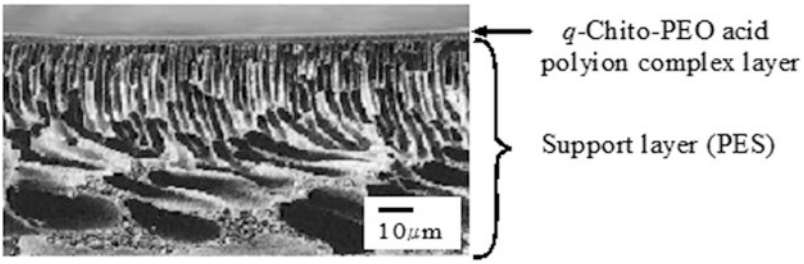


Fig. 5.19 Scanning electron microscope image of the cross-section of the *q*-Chito-PEO acid PIC-PES composite membrane

carboxylate groups in PEO acid and the quaternized ammonium groups in *q*-Chito (Scheme 5.4).

In every *q*-Chito-PEO acid PIC membrane, the separation factors for water permselectivity were increased by cross-linking *q*-Chito with PEO acids and reached a maximum at an equimolar ratio between the carboxylate and ammonium groups. The permeation rates were also affected by the molar ratios of the carboxylate and ammonium groups. With an increasing molecular weight of PEO acid, the separation factors at a molar ratio other than an equimolar ratio increased, but the separation factor at an equimolar ratio was not dependent on the molecular weight of PEO acid. This suggests that the PIC between the carboxylate and quaternized

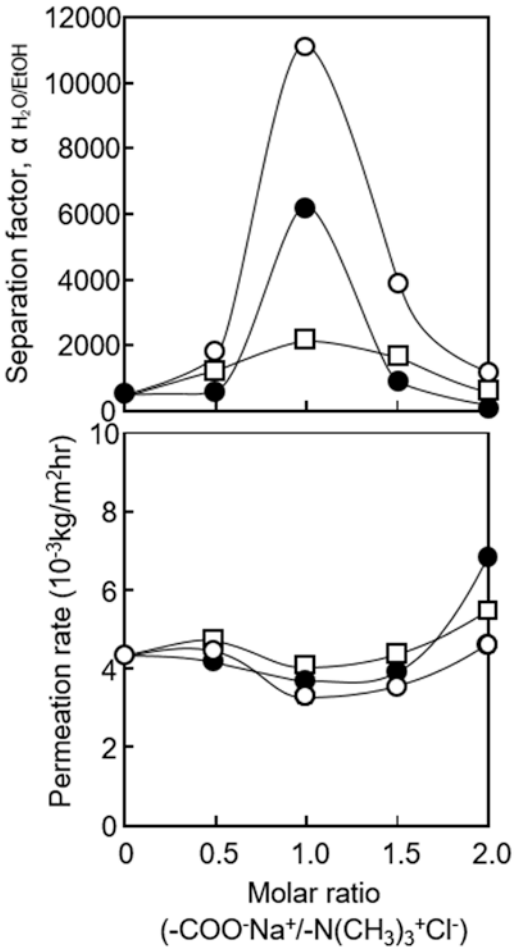
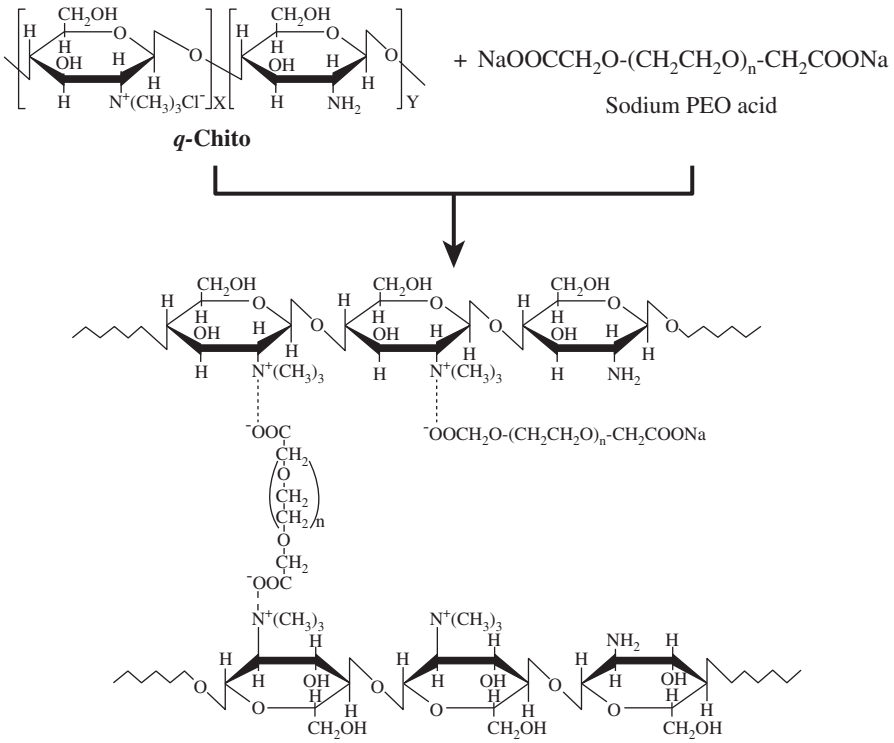


Fig. 5.20 Permeation and separation characteristics for an ethanol/water azeotrope through *q*-Chito-PEO acid PIC/PES composite membranes during evapomeation as a function of the molar ratio between the carboxylate groups in PEO acid and the ammonium groups in *q*-Chito. (●) PEO acid 400; (□) PEO acid 1000; (○) PEO acid 4000

ammonium groups was not formed stoichiometrically. Such difference in the PIC formation due to different PEO acid molecules yields significant differences in chemical and physical structure of the resulting membrane. Therefore, a competing factor may be significantly related to these results.

Huang reported that the pervaporation index, which is the product of the permeation rate and the separation factor, can be used as a measure of membrane performance during pervaporation. Because the membrane performance for evapomeation was estimated by the same method, the evapomeation index was also summarized in Table 5.2. The evapomeation index was the highest at a permeation temperature of 60 °C, and this suggests that there is an optimal temperature for the permeation of an ethanol-water azeotrope through the hydrophilic *q*-Chito-PEO acid PIC-PES composite membranes during evapomeation.



Scheme 5.4 PIC between *q*-chitosan (*q*-Chito) and sodium PEO acid

5.3.8 Technology of Temperature-Difference Controlled Evapomeation

Figure 5.21 shows the characteristics of permeation and separation for an aqueous DMSO solution through a chitosan membrane by the TDEV method (Uragami and Shinomiya 1992). In 21, the feed is an aqueous solution of 50 wt % DMSO, the temperature of the feed solution is kept constant at 40 °C and the temperature of membrane surroundings is changed to a temperature less than the temperature of the feed solution. Both the total permeation rate and separation factor increased with decreasing temperature of the membrane surroundings. The increase in the total permeation rate may be due to the increase of the solubility of vapor in the membrane with a drop of the temperature of the membrane surroundings according to Henry's law (Fig. 5.22).

The increase in the separation factor (i.e. an improvement of the permselectivity for water) is explained by the illustration shown in. 22. When the DMSO and water molecules which had vaporized from the feed mixture come close to the membrane surroundings, the DMSO vapour aggregates much easier than the water vapour (because the freezing points of DMSO and water are 18.4 °C and 0 °C respectively)

Table 5.2 Evapomeation characteristics and performance of *q*-chito-PEO acid 400 PIC/PES composite membrane for an ethanol-water azeotrope at various temperatures

Permeation temperature (°C)	Permeation rate × 10^{-2} (kg/(m^{-2} h^{-1}))			Separation factor $\alpha_{sep,\ H2O/EtOH}$	Sorption selectivity $\alpha_{sorp,\ H2O/EtOH}$	Diffusion selectivity $\alpha_{diff,\ H2O/EtOH}$	Evapomeation index
	total	H_2O	EtOH				
40	8.01	7.98	0.03	8000	850	9.4	640
60	35.12	34.96	0.16	6300	815	7.7	2205
80	91.03	87.60	3.43	800	727	1.1	728

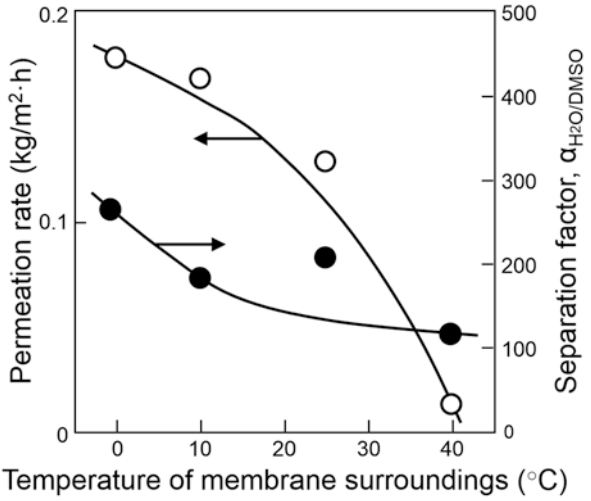


Fig. 5.21 Permeation rate and separation factor for an aqueous DMSO solution through the chitosan membrane as a function of the temperature of the membrane surroundings in TDEV. Feed solution: aqueous solution of 50 wt% DMSO, feed temperature: 40 °C

and tends to liquefy as the temperature of the membrane surroundings becomes lower. This aggregation of the DMSO molecules is responsible for the increase of permselectivity for water through the chitosan membrane. The increase in the separation factor with the TDEV method, in which the temperature of the membrane surroundings is lower than the temperature of the feed solution, is attributed to the influence of the degree of aggregation of the DMSO molecule on the membrane surroundings, which is significantly governed by the temperature of the membrane surroundings. The high water permselectivity of the chitosan membrane for aqueous DMSO solutions in TDEV is significantly enhanced by both a high affinity for water of the chitosan membrane and the decrease of the solubility selectivity for DMSO molecules into the chitosan membrane based on their aggregation on the membrane surroundings (Uragami 2008). Also TDEV characteristics for an aqueous solution of DMSO using a porous chitosan membrane were investigated and shown in Fig. 5.23.

The results in Fig. 5.23 can be explained by Fig. 5.24. When water and DMSO molecules, vaporized from the feed solution, come close to the membrane surroundings kept at lower temperature in TDEV, the DMSO vapor aggregates much easier than the water vapor, because the freezing point of DMSO molecules (18.5 °C) is

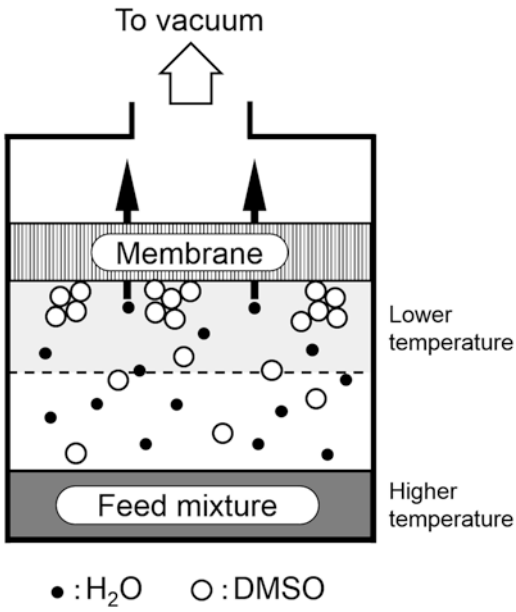


Fig. 5.22 Tentative mechanism of permeation and separation for aqueous DMSO solutions through the chitosan membrane by TDEV

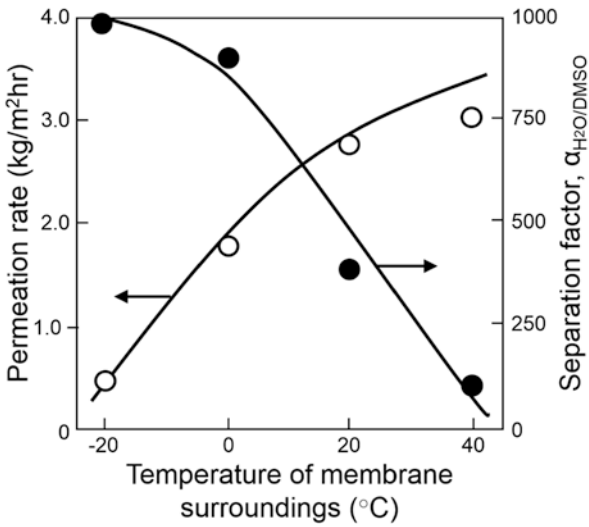


Fig. 5.23 Effect of the temperature of the membrane surroundings on the permeation rate and separation factor for the H_2O/DMSO selectivity of an aqueous solution of 50 wt% DMSO through a porous chitosan membrane during TDEV. Feed solution is 40 °C. Reduced pressure and inflow amount of dry are 2×10^4 Pa and 400 mL/min, respectively

much higher than that of water molecules (0 °C), and the aggregated DMSO molecules tend to be liquefied as the temperature of the membrane surroundings becomes lower. Both the aggregation of the DMSO molecules and the surface diffusion of the water molecules in the pores of hydrophilic chitosan membrane are responsible for the increase in the H_2O/DMSO selectivity through a porous chitosan membrane in TDEV.

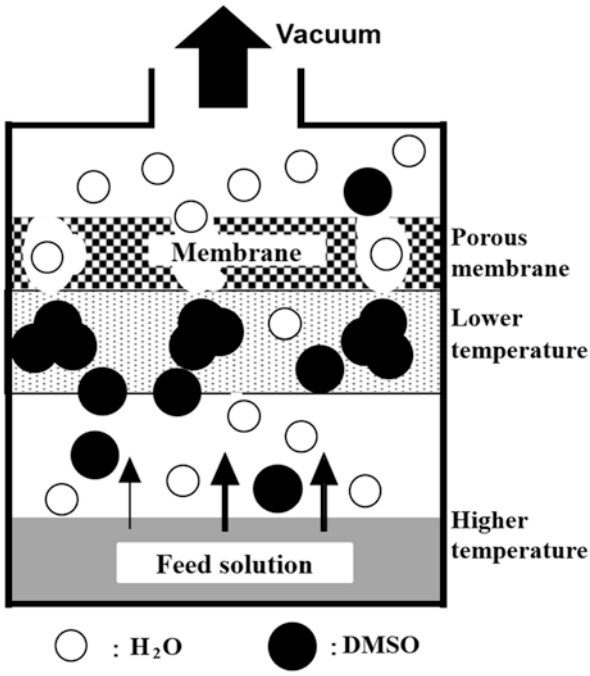


Fig. 5.24 Tentative mechanism of the permeation and separation characteristics for aqueous DMSO solution through a porous chitosan membrane

The results in Fig. 5.23 can be explained by Fig. 5.24. When water and DMSO molecules, vaporized from the feed solution, come close to the membrane surroundings kept at lower temperature in TDEV, the DMSO vapor aggregates much easier than the water vapor, because the freezing point of DMSO molecules (18.5 °C) is much higher than that of water molecules (0 °C), and the aggregated DMSO molecules tend to be liquefied as the temperature of the membrane surroundings becomes lower. Both the aggregation of the DMSO molecules and the surface diffusion of the water molecules in the pores of hydrophilic chitosan membrane are responsible for the increase in the H_2O/DMSO selectivity through a porous chitosan membrane in TDEV.

In TDEV, in which the temperature of the feed solution is kept constant and the temperature of the membrane surroundings is made less than the temperature of the feed solution, ethanol-permselectivity was observed as the temperature of the membrane surroundings was lowered (Uragami and Morikawa 1992). This was attributed to the fact that a solvent with a high freezing point can easily form aggregations in the membrane surroundings, which are kept at a low temperature. These aggregates permeate with difficulty through the membrane. For example, water molecules are easily aggregated then permeate slowly through the membrane.

5.3.9 Technology of Carrier Transport

The membranes prepared from mixtures of chitosan, poly(vinyl alcohol) (PV, and glutaraldehyde (GA) were insoluble in the acidic and the basic solution. These membranes were applied to the carrier transport for halogen ions (Uragami et al. 1983a, b). An example of the concentration change of Br ion and Na + ion and the pH changes in both the L side (basic side) and the R side (acidic side) with time due to the transport through the membrane is shown in.25, where the membrane was prepared from the chitosan/PVA ratio of 40/60 (wt%), the L side was 0.1 M NaBr in 0.1 M NaOH, and the A side 0.1 M HBr.

An example of the concentration change of Br^{-}ion and Na^{+} ion and the pH changes in both the L side (basic side) and the R side (acidic side) with time due to the transport through the membrane is shown in Fig. 5.25, where the membrane was prepared from the chitosan/PVA ratio of 40/60 (wt %), the L side was 0.1 M NaBr in 0.1 M NaOH, and the A side 0.1 M HBr.

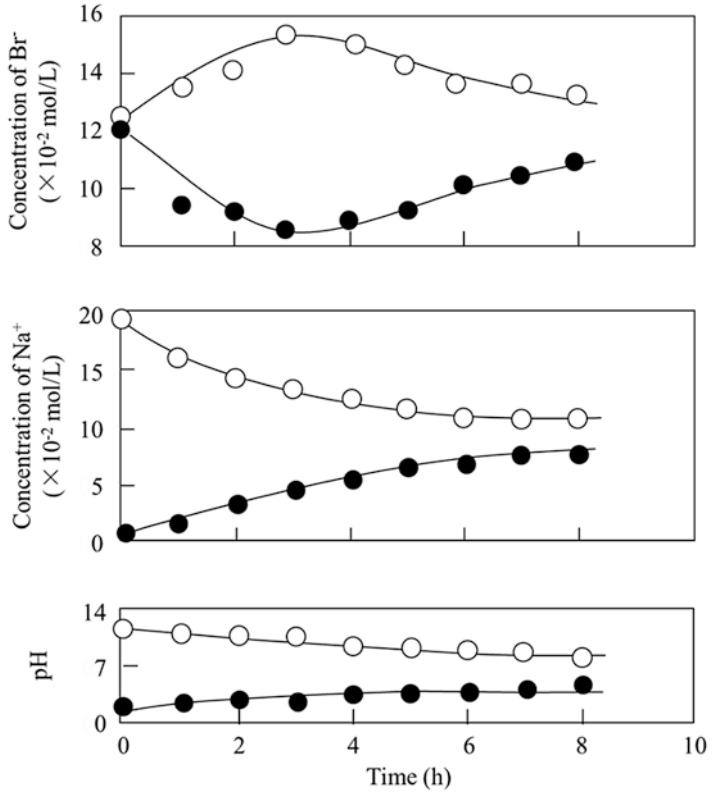


Fig. 5.25 Changes of the Br^{-} and the Na^{+} ion concentrations and pH with time on both sides through the membrane from the chitosan/PVA ratio of 40/60 L side: 0.1 M NaBr and 0.1 M NaOH; R side: 0.1 M HBr

The concentration of Br^- ion in the R side increased up to a maximum and then decreased with time. The concentration changes of Br^- ion in both sides were in the opposite direction. The increase of the Br^- ion concentration in the L side suggests that Br^- ions were actively transported across the membrane from the R side to the L side against its concentration gradient between both sides of the membrane because the initial concentration of Br^- ion was originally identical in both sides. The pH in the R side and the L side kept acidic and basic, respectively, for a long time. This result is attributed to the fact that the initial concentration of the OH^- ion in the L side is equal to that of the H^+ ion in the R side. The Na^+ ion concentration in the L side decreased with time caused by the diffusion through the membrane on the basis of its concentration gradient between both sides of the membrane. Since the membrane used in this work is an anion exchange membrane, it should be difficult to transport Na^+ ions across the membrane. However, Na^+ ions were transported from the L side to the R side across the membrane. This is due to the fact that the membrane is relatively open. In other words, this result also contains a possible mechanism that would counter diffuse Br^- ions in the L side transported actively across the membrane. Also, in the system of the Cl^-on and the I^-ion, similar results were obtained. It is expected that the transport of halogen ions in such system, where one side was acidic and the other basic, is significantly influenced by a pH difference between both sides and the diffusion of the counter cation.

The transport fraction and the transport rate of Br^- ion are calculated from Eqs. 5.2 and 5.3, respectively.

$$\text{Transport fraction}(\%) = \frac{\left[X^-\right]_{max} - \left[X^-\right]_0}{\left[X^-\right]_0} \times 100 \tag{5.2}$$

$$\text{Transport rate}\left(\text{mol}/\text{Lh}\,\text{cm}^2\right) = \frac{\left[X^-\right]_{max} - \left[X^-\right]_0}{At_{max}} \times 100 \tag{5.3}$$

where $[X^-]_0$ and $[X^-]_{max}$ are the initial and the maximum concentrations of the halogen ion in the L side, A is the membrane area t_{max} is the transport time for $[X^-]_{max}$, respectively.

These results under such conditions are caused by the fact that the pH in the L side and the R side are kept basic and acidic, respectively, for a long time. When the initial pH in the L side was lower or higher than 13.0, the pH in both sides became rapidly acidic or basic with time. These pH changes are attributed to a transport of H^+, OH^- and Na^+ ions, caused by a proton-jump mechanism, specific diffusion mechanism, and diffusive transport, respectively, as well as the transport of the Br^- ion caused by the uphill transport, in both sides. Consequently, both the transport fraction and the transport rate of the Br^-ion were smaller than those at pH 13.0. The permeation fraction of the Na^+ ion, determined by Eq. 5.4 from the L side to the R side through the membrane increased as the Na^+ ion concentration in the R side increased.

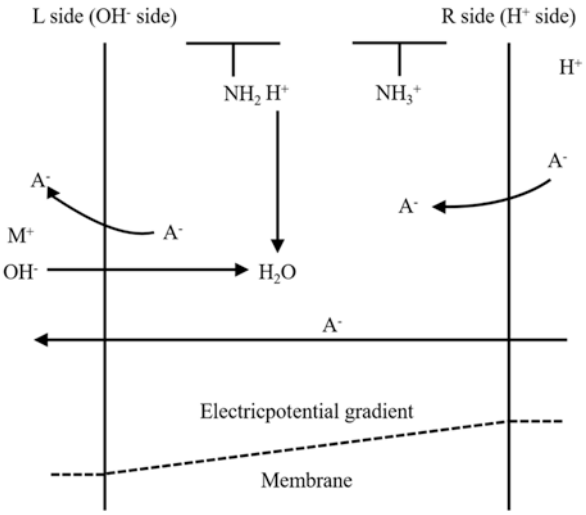


Fig. 5.26 Tentative mechanism for the uphill transport of halogen ions or organic anions the chitosan membrane. A^- is halogen ion or organic anion, M^+ is metal ion

$$\text{Permeation fraction}(\%) = \left(\left[Na^+\right]_{R,t} / \left[Na^+\right]_{L,0}\right) \times 100 \tag{5.4}$$

where

$[Na^+]_{L,0}$ the initial concentration of the Na^+ ion in the L side, $[Na]_{R,t}$ is the Na^+ ion concentration in the A side after t hours when the Br^- ion concentration in the L side is maximum.

Figure 5.26 is a tentative mechanism of the uphill transport of halogen ions.

In order to simplify the explanation, we instance the transport of the Br^- ion. When the Br^- ion is incorporated into the membrane on the A side (H^+ side), the hydrobromide is formed in the presence of hydrobromic acid and transferred through the membrane. As this hydrobromide reaches the L side (OH^- side), the hydrobromide changes to the amino group by neutralization and the Br^- ion is released. The released Br^- ion is transferred to the L side by the electric potential gradient between both sides. Consequently, it was suggested that the Br^- ion is actively transported through the membrane from the acidic side to the basic side.

If a greater pH difference and an electric potential difference between both sides could be kept for a long time, that is, the diffusive transport of metal ion from the L side to the R side should be prevented, the uphill transport of Br^- ion might be promoted. This expectation has been revealed by the result in Fig. 5.27, in which the uphill transport of the Cl^- ion through the chitosan membrane was promoted by trapping the metal ions with crown ether in the basic side in order to prevent the diffusion of metal ion from the basic side to the acidic side.

It was reported that an efficiency of the active transport of metal ions through cation exchange membranes was enhanced by an occurrence of a highly electrochemical potential gradient in the membrane (Uragami et al. 1983a, b; Wada et al. 1985). Therefore, the facilitation of the active transport of Cl^- ions in .27 is attrib-

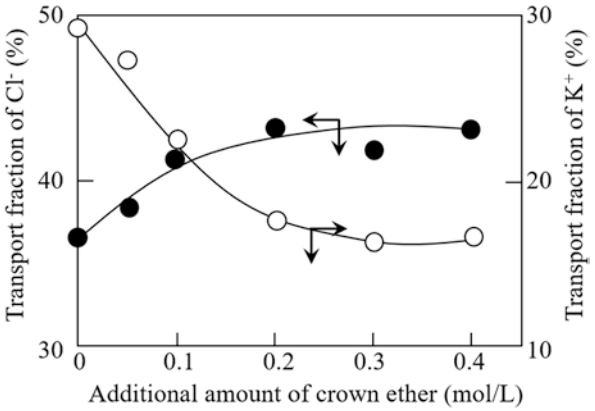


Fig. 5.27 Facilitation of the uphill transport of Cl^- ion through the chitosan membrane by adding of 18-crown-6 in the basic side

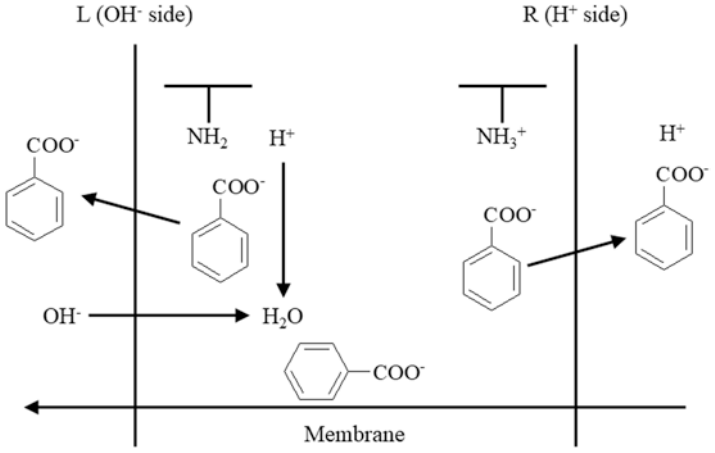


Fig. 5.28 Tentative mechanism for the transport of benzoate ions through the chitosan membrane against the concentration gradient

uted to an increase of the electrochemical potential gradient in the chitosan membrane based on a prevention of the diffusion permeation of K^+ ions with the addition of 18-crown-6 (Uragami et al. 1982). The chitosan membranes could actively transport benzoate ion and benzene sulfonate ion from the acidic side to the basic side against their concentration gradients as well as halogen ions, as shown in Fig. 5.28, and also *L*-phenyl alanine from the basic side to the acidic side against its

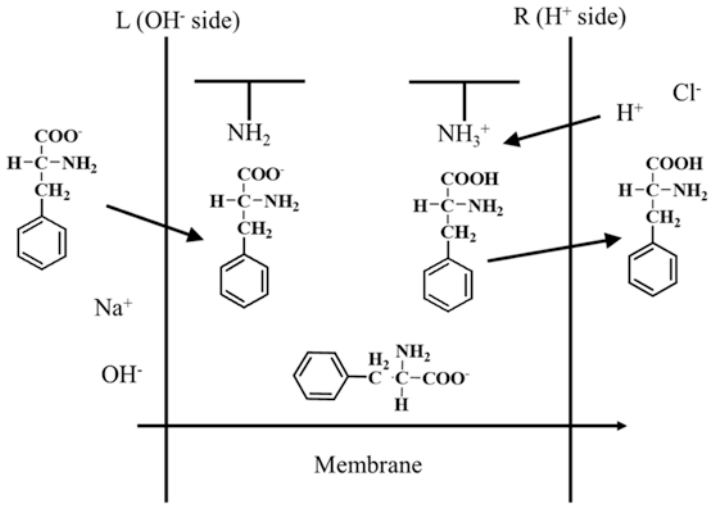


Fig. 5.29 Tentative mechanism for the transport of L-phnylalanine through the chitosan membrane against the concentration gradient

Table 5.3 Transport direction of uracil (Ura), cytosine (Cyt), adenine (Ade), guanine (Gua) and ion in the transport against the concentration gradient through the quaternized chitosan membrane

	Initial pH on L side			
Transported species	11.0	12.0	13.0	13.5
K^+	L → R	L → R	L → R	L → R
Ura (9.5)	L ← R	L ← R	L ← R	L ← R
Cyt (4.5, 12.2)	L → R	L → R	L → R	L ← R
Ade (4.15, 9.8)	L → R	L → R	L → R	L ← R
			L ← R	
Gua (3.2, 9.6, 12.4)	L → R	L → R	L → R	L ← R
			L ← R	

Arrows indicate the transport direction
Values in parentheses are pKa and pK_b values for nucleic acid bases
The initial pH on the R side was kept at 1.0 and the initial pH on the L side was changed

concentration gradient. The active transport is due to a tentative mechanism, as shown in Fig. 5.29 (Table 5.3).

A combination of the tentative mechanism in 28 and 29 could suggest that if the solution containing a mixture of amino acid and organic acid is added on both sides across the chitosan and if one side of the membrane is adjusted to acidic and the other to basic, an amino acid and an organic acid in the mixture could be actively transported against their concentration gradients through the chitosan from the basic side to the acidic side and vice versa, respectively, and consequently the amino acid and the organic acid from their mixture could be separated or concentrated by a cross-selective transport. Nucleic acid bases such as adenine, guanine, uracil, and

cytosine could also be actively transported against their concentration gradients through the chitosan and the direction of transport for them was significantly dependent on the pH or both sides. Quaternized chitosan cross-linked with ethylenglycol diglycidylether were applied to the uphill transport of nucleic acid bases. The transport results are summarized in Table 5.4. Uracil was transported against its concentration gradient from the basic side to the acidic side regardless of the pH on the basic side. Cytosine, adenine, and guanine were also transported against their concentration gradients, but the direction of their transport depended upon the pH on the basic side. In particular, transport directions for adenine and guanine were switched during identical transport experiments (Fig. 5.30).

5.3.10 Technology of Catalytically Function

A new method of enzyme immobilization by a polyion complex was proposed. An enzyme immobilizing membrane was prepared by ultrafiltrating a mixture consisted of quarternized chitosan, sodium polyacrylate and invertase in an aqueous NaBr solution as shown in the following sketch.

Table 5.4 Kinetic data in urea hydrolysis by the urease-immobilized membrane and native urease

Enzyme	K_m (10^2 M)	V_{max} (Ms^{-1})	$1/K_m$ (s^{-1})
Native urease	5.9	1.4×10^{-5}	16.9
Urease-immobilized membrane	4.3	9.5×10^{-3}	23.3

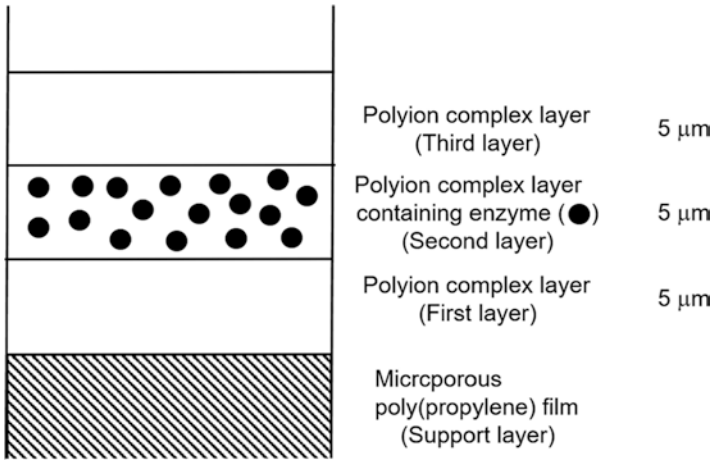


Fig. 5.30 Sketch of the enzyme immobilization polyion complex composite membrane

The permeation and hydrolysis characteristics of aqueous sucrose solution through the invertase immobilizing membrane was studied under some conditions. A hydrolysis rate of sucrose by the invertase immobilizing membrane corresponded to the Michael is-Menten type reaction (Uragami et al. 1986; Uragami and Aketa 1989). In Table 5.4 kinetic data for the hydrolysis of urea through the urease immobilizing membrane are compared with those of native urease in an aqueous urea solution. The value for the hydrolysis reaction of an aqueous urea solution through the urease immobilizing membrane was about 700 times that of the hydrolysis of urea by the native urease. These results could suggest that a continuous hydrolysis of substrates with permeation through the enzyme immobilizing membrane is possible.

Urease was covalently immobilized on glutaraldehyde-pretreated chitosan membranes (Krajewska et al. 1990). The optimum immobilization conditions were determined with respect to glutaraldehyde pretreatment of membranes and to reaction of glutaraldehyde-pretreated membranes with urease. The immobilized enzyme retained 94% its original activity. The properties of free and immobilized urease were compared. The Michaelis constant was about five times higher for immobilized urease than for the free enzyme, while the maximum reaction rate was lower for the immobilized enzyme. The stability of urease at low pH values was improved by immobilization; temperature stability was also improved. The optimum temperature was determined to be 65 °C the free urease and 75 °C for the immobilized form. The immobilized enzyme had good storage and operational stability and good reusability, properties that offer potential for practical application.

Jack bean urease was covalently immobilized on glutaraldehyde-pretreated chitosan membranes. Inhibition of the immobilized urease by boric acid was studied. Inhibition of chitosan-immobilized urease by boric acid was found to be competitive similar to that of the free enzyme.

Internally skinned polysulphone capillary membranes were coated with a viscous chitosan gel and used as an immobilization matrix for polyphenol oxidase. Bench-scale, single-capillary membrane bioreactors then were used to determine the influence of the chitosan coating on product removal after substrate conversion by immobilized polyphenol oxidase during the treatment of industrial phenolic effluents. The results indicate that greater efficiency was achieved in the removal of polyphenol oxidase-generated products by the chitosan membrane coating, as compared with chitosan flakes. This facilitated an increase in the productivity of the immobilized enzyme (Edwards et al. 1999).

The effect of phosphate buffer on the kinetic behavior of jack bean urease covalently immobilized on chitosan membrane was studied in the pH range 5.76–8.19, and compared with that of the free enzyme in an attempt to elucidate the effects of heterogeneity of the system on its kinetics. The chemical inhibition by the buffer, occurring between pH 5.76 and 7.50, was found to consist of two antagonistic effects: a decrease in the intrinsic enzyme activity and a reduction in the degree of environment-related inhibition. The apparent kinetic constants of the immobilized

urease: $v^{x}_{\max}$., K^{x}_{M} and $K_{i,buffer}$ exhibited both pH- and buffer concentration-dependence, anomalous as compared to the free enzyme: the optimum pH and the $pK_{i,buffer}$ values were displaced toward more acidic pH values, and the pK^{x}_{M} values were leveled off. The anomalies were gradually suppressed by increasing the buffer concentration. The anomalous behavior of chitosan membrane-immobilized urease was accounted for by a combined effect of: a) the increase in local pH on the membrane produced by both the enzymatic reaction and the electric charge of the support, and b) diffusional limitations imposed on substrate and product in the external solution.

Phenols are important industrial chemicals, and because they can be volatile, also appear as air pollutants. Wu et al. (2001) examined the potential of tyrosinase to react with the volatile phenol p-cresol. Three lines of evidence support the conclusion that volatile phenols react with tyrosinase and are coupled (i.e., chemisorbed) onto chitosan films. First, phenol-trapping studies indicated that p-cresol can be removed from vapors if the vapors are contacted with tyrosinase-coated chitosan films. Second, the ultraviolet absorbance of tyrosinase-coated chitosan films changes dramatically when they are contacted with cresol-containing vapors, whereas control films are unaffected by contacting with cresol vapors. Third, pressure measurements indicate that tyrosinase-coated chitosan films only react with cresol vapors if the oxygen cosubstrate is present. Additional studies demonstrate the potential of tyrosinase-coated chitosan films/membranes for the detection and removal of phenol vapors.

A protocol was used to prepare a dual-layer biomimetic membrane as support for enzyme immobilization by tethering chitosan on the surface of poly(acrylonitrile-*co*-maleic acid) (PANCMA) ultrafiltration hollow fiber membrane in the presence of 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC)/*N*-hydroxylsuccin-imide (NHS). The chemical change of the chitosan-modified PANCMA membrane surface was confirmed with Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy. Lipase from *Candida rugosa* was immobilized on this dual-layer biomimetic membrane using glutaraldehyde (GA), and on the nascent PANCMA membrane using EDC/NHS as coupling agent. The properties of the immobilized enzymes were assayed and compared with those of the free one. It was found that both the activity retention of the immobilized lipase and the amount of bound protein on the dual-layer biomimetic membrane (44.5% and 66.5 mg/m^2) were higher than those on the nascent PANCMA membrane (33.9% and 53.7 mg/m^2). The kinetic parameters of the free and immobilized lipases, K_m and V_{max}, were also assayed. The K_m values were similar for the immobilized lipases, while the V_{max} value of the immobilized lipase on the dual-layer biomimetic membrane was higher than that on the nascent PANCMA membrane. Results indicated that the pH and thermal stabilities of lipase increased upon immobilization. The residual activity of the immobilized lipase after 10 uses was 53% on the dual-layer biomimetic membrane and 62% on the nascent PANCMA membrane (Ye et al. 2005).

5.3.11 Technology of Gas Permeation

Chitosan-acetic acid complex membrane and several chitosan-polymer complex membranes have been prepared and the gas permeabilities of these membranes have been examined (Bai et al. 1988). It has been found that chitosan-acetic acid complex membrane shows high permselectivities for oxygen and carbon dioxide, and synthetic polymers can modify the permeation behavior of chitosan membrane for oxygen and carbon dioxide. The separation factor $\alpha_{CO2/O2}$ of these membranes were much smaller than unity, indicating possible applications for the preservations of fruits and vegetables. It has been noticed that the permeation behaviors of these membranes are markedly influenced by metal ions added into the membranes and the membranes have good mechanical strength.

The permeation and separation of carbon dioxide through a water-swollen chitosan membrane was studied. A chitosan membrane exhibited a large gas permeability when it was swollen by water vapor contained in the feed gas. Carbon dioxide preferentially permeated through the swollen chitosan membrane with a permeability of 2.5×10^{-8} $cm^3(STP)\cdot cm/(s\cdot cm^2\cdot cmHg)$ and a CO_2/N_2 separation factor of 70 at room temperature. This separation performance for CO_2 resulted from a basic property of the amino groups in the chitosan molecules. The membrane preparation method such as acetic acid concentration of the casting solution affected the membrane permeation rate. The effect of operation temperature was also measured. To increase the separation performance of the membrane, several methods of membrane treatment and operation were evaluated.

CO_2-selective membranes that obtain high CO_2 permeabilities accompanied with high CO_2/H_2 and CO_2/N_2 separation factors at industrial temperatures and pressures are applicable to fuel cell operations and flue gas purification. This paper describes the separation of carbon dioxide from a mixed gas stream of hydrogen and nitrogen by a chitosan membrane containing 40 wt % sodium arginate. Continuous membrane separations were done for a feed gas with 10% carbon dioxide, feed gas total pressures of 152 and 507 pa (1.5 and 5 atm), and temperatures ranging from 20 to 150 °C. The addition of arginine salts increases the number of amino groups for facilitated transport of CO_2 and increases the water levels in the arginine salt–chitosan membranes compared to swollen chitosan membranes at the same humidification conditions. At 152 pa (1.5 atm) feed pressure and 110 °C, there are maxima in the carbon dioxide permeabilities (1500 barrers), and the separation factors for CO_2/N_2 (852) and CO_2/H_2 (144). At higher pressure (507 pa (5 atm)), there were no maxima in the carbon dioxide permeabilities or separation factors, and there was less bound water in the membrane.

Extracorporeal CO_2 removal from circulating blood is a promising therapeutic modality for the treatment of acute respiratory failure. The enzyme carbonic anhydrase accelerates CO_2 removal within gas exchange devices by locally catalyzing HCO_3^- into gaseous CO_2 within the blood. In this work, Kimmel et al. (2013) covalently immobilized carbonic anhydrase on the surface of polypropylene hollow fiber membranes using glutaraldehyde activated chitosan tethering to amplify the density

of reactive amine functional groups for enzyme immobilization. XPS and a colorimetric amine assay confirmed higher amine densities on the chitosan coated fiber compared to control fiber. Chitosan/CA coated fibers exhibited accelerated CO_2 removal in scaled-down gas exchange devices in buffer and blood (115% enhancement vs. control, 37% enhancement vs. control, respectively). Carbonic anhydrase immobilized directly on hollow fiber membranes without chitosan tethering resulted in no enhancement in CO2 removal. Additionally, fibers coated with chitosan/carbonic anhydrase demonstrated reduced platelet adhesion when exposed to blood compared to control and heparin coated fibers.

The glutaraldehyde-crosslinked chitosan membrane (GXCM) was prepared and chiral resolution of (*R*, *S*)-2-amino-1-butanol (2A1B) was performed (Ingole et al. 2013). The membrane was analyzed by attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) for its chemical composition. The morphology of the membrane was studied by SEM and correlated with membrane performance. The performance of the GXCM membrane was quantified by performing chiral resolution of (*R*, *S*)-2-amino-1-butanol in pressure driven separation and the influences of permeation parameters such as operating pressure, concentration of feed solutions, concentration of copper (II) ions were investigated to understand the chiral selectivity of the membrane. The optical resolution of (*R*, *S*)-2-amino-1-butanol racemic mixture, 92% of enantiomeric excess (% ee) was achieved. The separation ability of the above crosslinked membrane was also investigated, and a separation factor of up to 5.6 was achieved.

A series of hydrogel membranes from chitosan (CS)/poly ether-block-amide (Pebax) blends were prepared and utilized for CO_2 separation. The membranes were characterized by field emission scanning electron microscope, FT-IR, DSC, XRD, positron annihilation lifetime spectroscopy, dynamic mechanical analyzer and tensile machine. The membranes exhibited high CO_2 permeabilities along with good operation stabilities. When the mass ratio of CS to Pebax was 1:1, the membrane showed the highest CO_2 permeability (2884 barrer) and a moderate separation factor (23.2 for CO_2/CH_4 and 65.3 for CO_2/N_2) at 85 °C. The high separation performance of these membranes was primarily arisen from the incorporation of Pebax. On one hand, when blending Pebax into CS, the increased fractional free volume led to more gas transport channels generated in polymer network, which directly increased CO_2 permeability. On the other hand, the increased fractional free volume also led to higher water content in membranes, which further increased CO_2 permeability. The effects of feed pressure and operating temperature on membranes properties were systematically investigated to further understand the transport mechanism in these membranes.

Mixed matrix membranes (MMMs) were prepared by incorporating organic surfactant-free hydrothermally synthesised ETS-10 and 1-ethyl-3-methylimidazolium acetate ionic liquid (IL) to chitosan (CS) polymer matrix. The membrane material characteristics and permselectivity performance of the two-component membranes were compared with the three-component membrane and the pure CS membrane. The addition of IL increased CO_2 solubility of the polymer, and, thus, the CO_2 affinity was maintained for the MMMs, which can be correlated

with the crystallinity, measured by FT-IR, and void fraction calculations from differences between theoretical and experimental densities. The mechanical resistance was enhanced by the ETS-10 nanoparticles, and flexibility decreased in the two-component ETS-10/CS MMMs, but the flexibility imparted by the IL remained in three-component ETS-10/IL/CS MMMs. The results of this work provide insight into another way of facing the adhesion challenge in MMMs and obtain CO_2 selective MMMs from renewable or green chemistry materials.

A facilitated transport mixed matrix membrane was fabricated by a surface coating method by Shen et al. (2015). Polyvinyl amine and chitosan were used as the polymer matrix materials and coated onto a porous polysulfone (PS) support. Graphene oxide (GO) grafted with hyperbranched polyethylenimine (HPEI-GO) was added as the nanofiller. The gas separation tests with CO_2/N_2 (10,90 v,v) mixed gas suggest that the addition of GO could improve CO_2/N_2 selectivity. The highest CO_2 permeance was 36 GPU in the membrane with 2.0 wt % HPEI-GO, and the optimal selectivity was 107 in the membrane with 3.0 wt % HPEI-GO. Herein, GO could provide a transport channel for CO_2 and enhance the long-term stability of the membranes. Further gas separation tests under various relative humidities confirmed that facilitated transport was the main mechanism of gas separation through the membrane. The stability test suggests that the membrane has long-term stability. CO_2 transports through the membrane mainly by the facilitated transport mechanism with assistance from the solution-diffusion mechanism, while N_2 transports only by the solution-diffusion mechanism.

CO_2 separation from CO_2/N_2 (20:80) gas mixture has been demonstrated by tetraethylenepentamine blended with chitosan (CS-TEPA) membrane (Prasad and Mandal 2017). Optimization of CS and TEPA weight ratio were carried out based on characterization details involving thermogravimetric analysis, Fourier transform infrared spectroscopy, X-ray diffraction, atomic force microscope, and field emission scanning electron microscope. Effects of water flow rate, pressure, and temperature were concurrently studied on CS-TEPA membranes through gas permeation. Almost twofold increase in CO_2 permeance (24.7 GPU) was detected in CS blend with 30% (w/w) of TEPA (CS70) as compared to pure CS membrane (12.5 GPU). CS70 yielded CO_2/N_2 selectivity of 80 whereas CS demonstrated a maximum of 54 at 90 °C. The membrane also exhibited improved stability at temperatures less than 120 °C which was evident from TGA isotherm trace. The proposed composite membrane can be a promising candidate for flue gas separation.

CO_2 separation was found to be facilitated by transport membranes based on novel chitosan (CS)-poly(styrene) (PS) and chitosan (CS)-poly(acrylonitrile) (PAN) copolymer matrices doped with methylimidazolium based ionic liquids: [bmim][BF_4], [bmim][PF_6], and [bmim][Tf_2N] (IL) (Otvagina et al. 2016). CS plays the role of biodegradable film former and selectivity promoter. Copolymers were prepared implementing the latest achievements in radical copolymerization with chosen monomers, which enabled the achievement of outstanding mechanical strength values for the CS-based membranes (75–104 MPa for CS-PAN and 69–75 MPa for CS-PS). Ionic liquid (IL) doping affected the surface and mechanical properties of

the membranes as well as the gas separation properties. The highest CO_2 permeability 400 Barrers belongs to CS-b-PS/[bmim][BF_4]. The highest selectivity α (CO_2/N_2) = 15.5 was achieved for CS-b-PAN/[bmim][BF_4]. The operational temperature of the membranes is under 220 °C.

Industrial polymeric membranes suffer from low CO_2 permeability as well as low gas pair selectivity. The presence of a suitable carrier in polymer matrix that can react reversibly with CO_2 can enhance the CO_2 permeability and CO_2-selective properties of polymeric membranes. The abundant amino groups of chitosan make it a good candidate for a CO_2 carrier. In this research, a new water-soluble amino derivative of chitosan was synthesized and characterized using FTIR, ^{1}H NMR, and elemental analysis. The synthesised chitosan derivative was blended with polyvinyl alcohol and used to prepare thin film composite (TFC) membranes for facilitated transport of CO_2 from a CO_2/CH_4 gas mixture. The effect of feed pressure, feed temperature and chitosan derivative content on the CO_2 permeance, CH_4 permeance and the CO_2/CH_4 selectivity of the prepared membranes were investigated. The new TFC membranes possessed acceptable CO_2 permeance and CO_2/CH_4 selectivity.

Chitosan/APTEOS mixed matrix membranes with 5, 10, 20 wt % loading of APTEOS were synthesized using solution casting method to improve gas separation properties of membranes (Zargar et al. 2017). Chitosan concentration was varied from 1 to 2.5 wt % to obtain best concentration of chitosan. CO_2 and N_2 permeabilities and CO_2/N_2 selectivity increased with feed pressure and APTEOS content up to 10 wt % and then decreased by the increase in APTEOS loading from 10 to 20 wt %. The membranes with 10 wt% content of APTEOS at 14 bar showed the best CO_2 permeability and CO_2/N_2 selectivity of 79.3 barrer and 84.38, respectively. FTIR and SEM results revealed appropriate distribution of nanoparticles in the polymeric matrix, and AFM analysis showed that the roughness of the membrane surface increased significantly by APTEOS content.

5.3.12 Technology of Fuel Cell

Chitosan membranes, cross-linked in sulphuric acid, were evaluated for methanol permeability at high to medium methanol concentrations and compared to the methanol permeability in Nafion 117 membranes. As a natural polymer, Chitosan is considered to be a cheap source of membrane material compared to Nafion. Methanol permeability through Chitosan membranes of medium molecular weight for methanol concentration of 12 mol/L at 20 °C was found to be $(8.0 \pm 0.5) \times 10^{-7}$ cm^2/s. This is almost three times lower than the permeability found for Nafion 117 of $(2.3 \pm 0.2) \times 10^{-6}$ cm^2/s at the same methanol concentration and temperature. An increased methanol concentration of 18.5 mol/L resulted in a decreased methanol permeability of $(5.2 \pm 0.6) \times 10^{-7}$ cm^2/s at 20 °C for Chitosan compared to increased methanol permeability of $(2.7 \pm 0.5) \times 10^{-6}$ cm^2/s at the same temperature for Nafion 117. An increased temperature of 50 °C at methanol concentration of 12 mol/L

however resulted in an order of magnitude increase in methanol permeability ($(4.1 \pm 0.3) \times 10^{-6}$ cm^2/s) for Chitosan membrane, which is comparable to the increase in methanol permeability of $(3.8 \pm 0.8) \times 10^{-6}$ cm^2/s for Nafion 117 membrane (Mukoma et al. 2004).

New polymer electrolyte composite membranes were prepared by using chitosan as the matrices and incorporating potassium hydroxide for ionic functionality. These membranes had a three-layer structure, which consisted of a porous intermediate layer and two crosslinked solid surface layers. Their ionic-conductive properties were investigated using impedance spectroscopy. Some composite membranes showed a conductivity near 10^{-2} S/cm after hydration for 1 h at room temperature. Several composite membranes were preliminarily integrated into fuel cells for the assessment of their electrochemical performance using hydrogen as fuel, air as oxidant and platinum as the electrode catalysts. A membrane electrode assembly was fabricated by directly pressing two gas-diffusion electrodes onto the two opposite surfaces of the composite membrane. All fuel cells showed an open-circuit potential around 1.0 V, and under appropriate running conditions, a current density of about 30 mA/cm^2 was achieved. Some possible improvements on the performance of the resultant fuel cells are also suggested (Wan et al. 2006).

A series of blending chitosan sulfate membranes have been developed by grafting the chitosan monomers with sulfonic groups, then cross-linking the polymers from the bond reactions between the sulfonic groups in the chitosan sulfate and the amido groups in the pure chitosan monomers. Mechanical characterizations demonstrated that the dimensional swelling as well as the methanol crossover of the chitosan membranes were suppressed successfully by the polymer blending, with area swelling value decreased from 55.1% to 39.3% and methanol diffusion coefficient decreased from 1.0×10^{-6} cm^2/s of pure chitosan to 4.7×10^{-7} cm^2/s of the membrane with chitosan sulfate content of ~9.1 wt % (CCSM 110). The thermal analysis indicated that the blending chitosan sulfate membranes were structure stable below 100 °C. The blending membrane showed the best conductivity (0.03 S/cm at 80 °C). The methanol permeability of CCSM 110 was much lower compared with that of Nafion 112 (1.9×10^{-6} cm^2/s).

As inorganic proton conductors, phosphomolybdic acid (PMA), phosphotungstic acid (PWA) and silicotungstic acid (SiWA) are extremely attractive for proton-conducting composite membranes. An interesting phenomenon has been found in previous experiments by Cui et al. (2009) that the mixing of chitosan (CS) solution and different heteropolyacids (HPAs) leads to strong electrostatic interaction to form insoluble complexes. These complexes in the form of membrane (CS/PMA, CS/PWA and CS/SiWA composite membranes) have been prepared and evaluated as novel proton-conducting membranes for direct methanol fuel cells. Therefore, HPAs can be immobilized within the membranes through electrostatic interaction, which overcomes the leakage problem from membranes. CS/PMA, CS/PWA and CS/SiWA composite membranes were characterized for morphology, intermolecular interactions, and thermal stability by SEM, FTIR, and TGA, respectively. Among the three membranes, CS/PMA membrane was identified as ideal for DMFC as it

exhibited low methanol permeability (2.7×10^{-7} cm^2/s) and comparatively high proton conductivity (0.015 S/cm at 25 °C) (Cui et al. 2009).

A series of quaternized-chitosan derivatives (QCDs) with various degrees of quaternization was synthesized using glycidyltrimethylammonium chloride as a main quaternized reagent. These QCDs were then processed into hydroxide—form quaternary ammonium salts with aqueous potassium hydroxide solutions. The resultant hydroxide form QCD gels were further crosslinked into anion-exchange membranes using ethylene glycol diglycidyl ether. The crosslinking density, crystallinity, swelling index, ion exchange capacity, ionic conductivity and thermal stability of the crosslinked membranes were subsequently investigated. It was found that properties of crosslinked membranes were modulated mainly by the degree of quaternization and crosslinking density of membranes. Some membranes exhibited promising characteristics and had the potential for applications in alkaline polymer electrolyte fuel cells in considering their integrative properties (Wan et al. 2010).

The chitosan flakes were prepared into membranes and the Chs membranes were modified by cross-linking with H_2SO_4. The cross-linked Chs membranes were characterized for the application in direct methanol fuel cells. The Chs membrane characteristics such as water uptake, thermal stability, proton resistance and methanol permeability were compared to that of high performance conventional Nafion 117 membranes. Under the temperature range studied 20–60 °C, the membrane water uptake for Chs was found to be higher than that of Nafion. Thermal analysis revealed that Chs membranes could withstand temperature as high as 230 °C whereas Nafion 117 membranes were stable to 320 °C under nitrogen. Nafion 117 membranes were found to exhibit high proton resistance of 284 s/cm than Chs membranes of 204 s/cm. The proton fluxes across the membranes were 2.73 mol/cm^2 s for Chs- and 1.12 mol/cm^2 s Nafion membranes. Methanol permeability through Chs membrane was less, 1.4×10^{-6} cm^2/s for Chs membranes and 3.9×10^{-6} cm^2/s for Nafion 117 membranes at 20 °C. Chs and Nafion membranes were fabricated into membrane electrode assemblies (MAE) and their performances measure in a free-breathing commercial single cell DMFC. The Nafion membranes showed a better performance as the power density determined for Nafion membranes of 0.0075 W/cm^2 was 2.7 times higher than in the case of Chs MEA (Osifo and Masala 2010).

A novel double layer proton exchange membrane (PEM) comprising a layer of structurally modified chitosan, as a methanol barrier layer, coated on Nafion®112 was prepared and assessed for direct methanol fuel cell (DMFC) applications (Mahdi Hasani-Sadrabadi et al. 2011). SEM micrographs of the designed membrane revealed a tight adherence between layers, which indicate the high affinity of opposite charged polyelectrolyte layers. Proton conductivity and methanol permeability measurements showed improved transport properties of the designed membrane compared to Nafion®117. Moreover, DMFC performance tests revealed a higher open circuit voltage and power density, as well as overall fuel cell efficiency for the double layer membrane in comparison with Nafion®117, especially at elevated methanol solution feed. The obtained results indicate the designed double layer membrane as a promising PEM for high-performance DMFC applications.

A novel triple-layer proton exchange membrane comprising two thin layers of structurally modified chitosan, as methanol barrier layers, both sides coated with Nafion®105 is prepared and tested for high-performance direct methanol fuel cell applications (Mahdi Hasani-Sadrabadi et al. 2012). A tight adherence is detected between layers from SEM and EDX data for the cross-sectional area of the newly designed membrane, which are attributed to high affinity of opposite charged polyelectrolyte layers. Proton conductivity and methanol permeability measurements show improved transport properties for the multi-layer membrane compared to Nafion®117 with approximately the same thickness. Moreover, direct methanol fuel cell tests reveal higher open circuit voltage, power density output, and overall fuel cell efficiency for the triple-layer membrane than Nafion®117, especially at concentrated methanol solutions. A power output of 68.10 mW/cm^2 at 5 M methanol feed is supplied using multi-layer membrane, which is found to be about 72% more than that of for Nafion®117. In addition, fuel cell efficiency for multi-layer membrane is measured about 19.55% and 18.45% at 1 and M methanol concentrations, respectively. Owing to the ability to provide high power output, significantly reduced methanol crossover, ease of preparation and low cost, the triple-layer membrane under study could be considered as a promising polyelectrolyte for high-performance direct methanol fuel cell applications.

Fuel cell is an electrochemical device which converts chemical energy stored in a fuel into electrical energy. Fuel cells have been receiving attention due to its potential applicability as a good alternative power source. Recently, cost-effective and eco-friendly biopolymer chitosan has been extensively studied as a material for membrane electrolytes and electrodes in low to intermediate temperature hydrogen polymer electrolyte fuel cell, direct methanol fuel cell, alkaline fuel cell, and biofuel cell. This paper reviews structure and property of chitosan with respect to its applications in fuel cells. Recent achievements and prospect of its applications have also been included.

Sulfonated graphene oxide (SGO) nanosheets with controllable sulfonic acid group loading are synthesized via the facile distillation–precipitation polymerization, and then incorporated into chitosan (CS) matrix to prepare nanohybrid membranes. The microstructure and physicochemical properties of the resulting membranes are extensively investigated. Compared with CS control and GO-filled membranes, SGO-filled membranes attain enhanced thermal and mechanical stabilities due to the strong electrostatic attractions between $–SO_3H$ of SGO and $–NH_2$ of CS, which inhibit the mobility of CS chains. Additionally, the inhibited mobility reduces the area swellings of SGO-filled membranes, reinforcing their structural stabilities. The incorporation of SGO generates acid–base pairs along CS–SGO interface, which work as facile proton-hoping sites and thus construct continuous and wide proton transfer pathways, yielding enhanced proton conductivities under both hydrated and anhydrous conditions. Meanwhile, the conductivity can be elevated by increasing the sulfonic acid group loading and content of SGO. Particularly, incorporating 2.0% S4GO can afford the nanohybrid membrane a 122.5% increase in hydrated conductivity and a 90.7% increase in anhydrous conductivity when compared with CS control membrane. The superior conduction properties then

offered a significant enhancement in H_2/O_2 cell performances to the nanohybrid membranes, guaranteeing them to be promising proton exchange membranes.

A proton conducting polymer electrolyte nanocomposite membrane has been fabricated by using (PVA, chitosan, and poly (styrene sulfonic acid) (PSSA) polymers and montmorillonite Cloisite®30B clay with the objective of its application in direct methanol fuel cells. Comparative studies of a PVA/PSSA/Chitosan/Cloisite30B clay composite membrane with a base PVA/PSSA membrane has been carried out by using thermal gravimetric analysis, DSC, dynamic mechanical analysis, X-ray diffraction, methanol permeability and proton conductivity measurements. Properties of the membrane have been compared with Nafion®117 at identical test conditions. Methanol permeability of the PVA/PSSA/Chitosan/Cloisite30B clay composite membrane has been found to be superior to that of PVA/PSSA as well as Nafion117 membranes. Water uptake of the membrane is much higher compared to the Nafion117 membrane. Proton conductivity of the membrane has been found in the range of 10^{-2} S/cm at room temperature and 70% relative humidity. Selectivity of the membrane is in the range of 10^4 S s/cm^3, which is better than that of Nafion117 (Gaur et al. 2014).

Currently, enhancing the proton conductivity is one challenge for chitosan membrane to work as proton exchange membrane for direct methanol fuel cell. In this study, halloysite nanotubes bearing sulfonate polyelectrolyte brushes (SHNTs) are synthesized via distillation–precipitation polymerization and then incorporated into chitosan matrix to fabricate nanohybrid membranes (Bai et al. 2014). The membranes are characterized using field emission scanning electron microscope, fourier transform infrared, thermogravimetric analysis, differential scanning calorimetry, and mechanical tester. It is found that SHNTs generate strong electrostatic attractions to chitosan chains, which inhibit the chain mobility and thus enhance the thermal and mechanical stabilities of nanohybrid membranes. The results of water uptake, area swelling, proton conductivity, and activation energy reveal that the high aspect nanotube and long polyelectrolyte brush allow SHNTs to construct continuous and wide pathways, along which sulfonic acid–amide acid–base pairs are formed and work as low-barrier proton-hoping sites, imparting an enhanced proton transfer via Grotthuss mechanism. In such a way, the proton conductivity of CS membrane is obviously enhanced, and 15% SHNTs can afford a 60% enhancement in conductivity to the nanohybrid membrane, particularly. Moreover, the methanol permeability and selectivity of the as-prepared membranes are investigated in detail.

Novel alkaline anion-exchange membranes composed of chitosan (CS) and 1-ethenyl-3-methyl-1H-imidazoliumchloride polymer with 1-ethenyl-2-pyrrolidone (abbreviated as EMImC-Co-EP) are prepared by a combined thermal and chemical cross-linking technique. The hydroxide conductivity (σ_{OH^-}), water uptakes, ion exchange capacity, thermal stability, mechanical property, oxidative stability and alkaline stabilities of CS/EMImC-Co-EP membranes are measured to evaluate their applicability in H_2/O_2 alkaline fuel cells. The effects of thermal cross-linking temperature and membrane composition on membrane OH^- conductivity are studied using AC impedance technique. FTIR, SEM and TG analysis are

used for structural characterization of these membranes. It is found that the OH^- conductivity of the membranes increases with temperature and exceeds 10^{-2} S/cm at 80 C. The CS/EMImC-Co-EP membranes show excellent thermal stability with onset degradation temperature high above 200 °C. In particular, the high alkaline stability is achieved for the CS/EMImC-Co-EP membranes in hot 8.0 M KOH at 85 °C without losing their integrity and OH^- conductivity during 300 h of evaluation, and also a relatively high oxidative stability. The membrane electrode assembly (MEA) fabricated with CS/EMImC-Co-EP (1:0.5 by mass) gives an initial power density of 21.7 mW/cm^2 using H_2 as the fuel and O_2 as oxidant at room temperature, on a low metal loading on both the anode and the cathode of 0.5 mg (Pt)/cm^2 at ambient temperature.

A novel polymer composite electrolyte consisting of quaternized polyvinyl alcohol (q-PVA) and quaternized chitosan nano-particles (q-chitosan) is synthesized for use in a direct alcohol alkaline fuel cell (DAAFC). The quaternization efficiencies of PVA and chitosan particles are 2.6% and 39%, respectively. Incorporation of 5% q-chitosan nano-particles into the q-PVA matrix results not only in an increased ion-exchange capacity but also a decreased polymer crystallinity and higher free volume hole density, which significantly enhance ion conduction. The shrunk polymer free volume size suppresses methanol permeability in the q-PVA/Q-chitosan composite. In addition, the resulting nanocomposite exhibited an inhibited in-plane swelling ratio without sacrificing the alkali uptake level. The KOH-doped q-PVA/Q-chitosan was resistant to the Fenton reagent. In DAAFCs, a maximum power density of 73 mW/cm^2 is achieved using the q-PVA/5% Q-chitosan electrolyte when fed with 2 M methanol at 60 °C or 59 mW/cm^2 with 3 M ethanol at 60 °C. The resulting maximum power densities are higher than most literature data. The alkaline fuel cell demonstrated stable long-term power output for at least 230 h. This result confirms that the q-PVA/q-chitosan electrolyte exhibits great potential for future commercialization (Liao et al. 2015).

The major problems of polymer electrolyte membrane fuel cell technology that need to be highlighted are fuel crossovers (e.g., methanol or hydrogen leaking across fuel cell membranes), CO poisoning, low durability, and high cost. Chitosan and alginate-based biopolymer membranes have recently been used to solve these problems with promising results. Current research in biopolymer membrane materials and systems has focused on the following: (1) the development of novel and efficient biopolymer materials; and (2) increasing the processing capacity of membrane operations. Consequently, chitosan and alginate-based biopolymers seek to enhance fuel cell performance by improving proton conductivity, membrane durability, and reducing fuel crossover and electro-osmotic drag. There are four groups of chitosan-based membranes (categorized according to their reaction and preparation): self-cross-linked and salt-complexed chitosans, chitosan-based polymer blends, chitosan/inorganic filler composites, and chitosan/polymer composites. There are only three alginate-based membranes that have been synthesized for fuel cell application. This work aims to review the state-of-the-art in the growth of chitosan and alginate-based biopolymer membranes for fuel cell applications.

Phosphonic acid (PA) groups, as one kind of feasible proton carrier, possess the distinct intrinsic proton conduction ability and have triggered intensive attention in proton conducting materials (Bai et al. 2015). In this study, phosphorylated graphene oxide (PGO) nanosheets are incorporated into chitosan (CS) matrix to prepare nanohybrid membranes. The microstructure and physicochemical properties of PGO and the membranes are investigated systematically. The grafted polymer layer is found to be about 26 wt % of PGO, which considerably increases the ion exchange capacity from 0.44 mmol/g of GO to 0.79 mmol/g. Compared with CS control and GO-filled membranes, PGO-filled membranes achieve higher thermal and mechanical stabilities due to the strong electrostatic interactions between PGO (–PO_3H) and CS (–NH_2). PGO provides efficient hopping sites (–PO_3H, –$PO_3^{-}\cdots{}^{+}{}_3HN$–), which allow the formation of highly conductive channels along PGO surface. These channels are found to significantly facilitate proton conduction under both hydrated and anhydrous conditions. Particularly, nanohybrid membrane with 2.5% PGO acquires a 22.2-time increase in conductivity from 0.25mS/cm to 5.79 mS/cm (160 °C, 0% RH). With this benefit, the hydrogen fuel cell using PGO-filled membranes displays much higher cell performance than those using CS control and GO-filled membranes.

Free-standing Chitosan/phosphotungstic acid polyelectrolyte membranes were prepared by an easy and fast in-situ ionotropic gelation process performed at room temperature. Scanning electron microscopy was employed to study their morphological features and their thickness as a function of the chitosan concentration. The membrane was tested as proton conductor in low temperature H_2–O_2 fuel cell allowing to get peak power densities up to 350 mW/cm^2. Electrochemical impedance measurements allowed to estimate a polyelectrolyte conductivity of 18 mS/cm (Santamaria et al. 2015).

The major problems of polymer electrolyte membrane fuel cell technology that need to be highlighted are fuel crossovers (e.g., methanol or hydrogen leaking across fuel cell membranes), CO poisoning, low durability, and high cost. Chitosan and alginate-based biopolymer membranes have recently been used to solve these problems with promising results. Current research in biopolymer membrane materials and systems has focused on the following: 1) the development of novel and efficient biopolymer materials; and 2) increasing the processing capacity of membrane operations. Consequently, chitosan and alginate-based biopolymers seek to enhance fuel cell performance by improving proton conductivity, membrane durability, and reducing fuel crossover and electro-osmotic drag. There are four groups of chitosan-based membranes (categorized according to their reaction and preparation): self-cross-linked and salt-complexed chitosans, chitosan-based polymer blends, chitosan/inorganic filler composites, and chitosan/polymer composites. There are only three alginate-based membranes that have been synthesized for fuel cell application. This work aims to review the state-of-the-art in the growth of chitosan and alginate-based biopolymer membranes for fuel cell applications.

Using 1-aminoanthraquinone, cyanuric chloride, dimethyl propylene diamine, diethyl sulfate as reactants, a reactive cationic dye (RCD) was synthesized. Then the

dye prepared was used to modify the pristine chitosan (CTS) membrane to synthesize a novel alkaline exchange membrane for low temperature fuel cells. The performance of the prepared membranes were researched in details. FTIR and SEM were used for chemical and structural characterization of the membranes while thermo gravimetric analysis was adopted to study the membranes' thermal stability. The properties of the membranes such as OH^- conductivity (σ), water uptake (WU), ion exchange capacity (IEC) and mechanical property were also investigated systematically to evaluate their application performances. The results showed that the OH^- conductivity of the membrane (52 μm) can reach 4.59×10^{-3} S/cmand increase with the temperature increasing from 30 °C to 80 °C. After immersing the CTS/RCD membrane (52 μm) in the KOH solution (80 °C, 8 mol/L) for 300 h, the OH^- conductivity of the membrane didn't decrease, on the contrary, it increased to 1.057×10^{-2} S/cm, which indicated that the membrane had the excellent stability to resist the strong alkaline lye (Wang et al. 2016).

Silica-coated carbon nanotubes (SCNTs), which were obtained by a simple sol–gel method, were utilized in preparation of chitosan/SCNTs (CS/SCNTs) composite membranes. The thermal and oxidative stability, morphology, mechanical properties, water uptake and proton conductivity of CS/SCNTs composite membranes were investigated. The insulated and hydrophilic silica layer coated on CNTs eliminates the risk of electronic short-circuiting and enhances the interaction between SCNTs and chitosan to ensure the homogenous dispersion of SCNTs, although the water uptake of CS/SCNTs membranes is reduced owing to the decrease of the effective number of the amino functional groups of chitosan. The CS/SCNTs composite membranes are superior to the pure CS membrane in thermal and oxidative stability, mechanical properties and proton conductivity. The results of this study suggest that CS/SCNTs composite membranes exhibit promising potential for practical application in proton exchange membranes (Liu et al. 2016).

Herein, a series of quaternized halloysite nanotubes (QHNTs) bearing different imidazoliums groups are prepared *via* distillation–precipitation polymerization and quaternarization, and then embedded into chitosan matrix to fabricate hybrid membranes. Systematic characterizations and measurements are performed to explore the relationships between the ligand of ammonium and the performance of hybrid membrane. It is found that QHNTs are well-dispersed within CS matrix, and the chain mobility of CS is promoted driven by repulsive interactions from QHNTs, in turn affording the increments of water uptake and area swelling. Together with the promoted chain mobility, low-barrier conduction pathways are formed along the QHNTs surface and then confer a significant enhancement in hydroxyl conductivity to hybrid membranes. For QHNTs, hydrophilic ligand could adsorb more water molecules and short ligand is more favorable for contacting with hydroxyl, both of which are beneficial for high hydroxyl conductivity. Particularly, incorporating 7.5% QHNTs with ester-type QA ligand endows the hybrid membrane with an 89% increase in conductivity from 0.009 to 0.017 S/cm at 90 °C and 100% RH. Yet under anhydrous conditions, the positive charge dispersity of QA and stereo-hindrance effect of the ligand are the predominant factors for hydroxyl ion hopping (Shi et al. 2016).

Chitosan has been attracting considerable interest as polymer electrolyte in fuel cells. However, proton conductivity of chitosan is low and it is necessary to enhance

its conductivity. In this work, 10 wt % sulfonated chitosan (SCS) and different amounts of sulfonated graphene oxide (SGO) nanosheets are incorporated into a chitosan membrane to investigate their effects on the electrochemical properties of the membrane. The proton conductivity and methanol permeability tests conducted on the CS/SCS/SGO membranes show that the conductivity is increased by 454%, the permeability is reduced by 23% and hence the selectivity is increased by 650%, relative to the neat chitosan, at SGO content of 5 wt %. Furthermore, combined addition of SCS and SGO to chitosan causes much more proton conductivity enhancement than the individual additives due to the synergistic effect of SCS and SGO. The observed synergistic effect reveals the importance of the chemical functionality of chitosan and nanofillers in the formation of ionic cluster domains with enhanced size within the membranes for proton transport. Finally, a Nernst–Planck based model is applied to the experimental proton conductivity data in order to shed more light on the role of GOs in the proton conductivity mechanism of chitosan (Shirdast et al. 2016).

Modified chitosan membrane was synthesized as a proton exchange membrane for microbial fuel cell application. Glutaraldehyde and sulfosuccinic acid were used as crosslinking agents in order to improve its ultimate tensile strength and proton conductivity. 3-Chloro-2-hydroxypropyl trimethylammonium chloride was employed for quaternization to develop its antimicrobial activity. The results showed that the proton conductivity of the membrane was enhanced with the content of sulfosuccinic acid, as a result of proton carrier sites, until a certain value was reached. The additional positive charge from quaternization increased with the reaction time. The morphological change of microorganisms in contact with the surface of the quaternized chitosan membrane exhibited damage and the number of damaged microorganisms increased with the positive charge density; nevertheless, the high positive charge density resulted in not only a high antimicrobial property, but also in significant water uptake of the quaternized chitosan membrane. As a consequence, the strength of the membrane was lost. Additionally, the positive charge also accelerated the adhesion of microorganisms at the membrane surface, but the surface growth could be retarded due to the high number of microorganisms being damaged.

Polymer electrolyte membranes based on organic–inorganic nanocomposites consisting of chitosan and silica supported silicotungstic acid (IHPA) are developed using simple solution casting technique. Compared to pristine chitosan membranes, IHPA incorporated membranes exhibit higher thermal, mechanical, oxidative stabilities and better membrane selectivity due to the strong electrostatic interaction and hydrogen bonding between the polymer, cross linking agent and IHPA nanoparticles. IHPA provides efficient hopping sites which allow the formation of conducting channels, which thereby facilitates the proton conduction under both dry and hydrated conditions significantly. Nanocomposite membrane filled with 5 wt IHPA has the highest proton conductivity of 9.0×10^{-3} S/m at 100 °C. All the prepared CS-IHPA nanocomposite membranes exhibit better stability against oxidative degradation, water uptake and retention ability, proton conductivity and membrane selectivity. CS-IHPA-5 membrane exhibits the best overall performance as a mem-

brane, which is mainly due to the strong interactions between chitosan matrix and IHPA. CS-IHPA-3 membrane shows high OCV (0.73 V) and maximum power density (54.2 mW/cm^2) compared to the other membranes. The present study provides a promising strategy for the design and fabrication of high performance polymer electrolyte membranes for fuel cell applications, which is cost effective and eco-friendly (Vijayakumar and Khastgir 2018).

5.3.13 Technology of Optical Resolution

For the optical resolution of α-amino acids, enantioselective membranes cross-linked were prepared using sodium alginate and chitosan as membrane materials. The characteristics of the membranes in terms of their chemical structure and swelling indices were analyzed. The permselective properties of the membranes in the separation of optical isomers of α-amino acids were studied using aqueous solutions of tryptophan and tyrosine as feed solutions. The employed membrane process was a pressure driven process using a low Δp such as 1 or 2 kgf/cm^2. Effects of degree of crosslinking, feed concentration, operating pressure and different kinds of feed solutions on the membrane performances were studied. When a chitosan membrane with 70% of swelling index was used for the optical resolution of tryptophan racemic mixture (0.49 mmol/l aqueous solution), over 98% of enantiomeric excess (e.e.) and 6.4 mg/(m^2 h) of flux were obtained.

Ultrafiltration experiments for the chiral separation of racemic phenylalanine were performed with DNA-immobilized chitosan membranes having various pore sizes. Atomic analysis on the membranes showed that the chitosan membranes covalently bound six times more DNA than the cellulose membranes used in our previous study (Higuchi et al. 2003). Phenylalanine preferentially permeated through DNA-immobilized chitosan membranes with a pore size <6.4 nm [molecular weight cut-off (MWCO) < 7000]. The binding affinity of a specific enantiomer due to the pore size of the DNA-immobilized membranes regulated the preferential permeation of the enantiomer through the membranes. L-Phenylalanine was adsorbed on the DNA-immobilized chitosan membranes with a pore size <6.4 nm (MWCO <67,000), while there was little difference between the adsorption of D-phenylalanine and L-phenylalanine on the membranes with a pore size >6.4 nm (MWCO >67,000). The DNA-immobilized chitosan membranes were categorized as channel type membranes.

Semi-interpenetrating networks (IPN)-structured chitosan/β-cyclodextrin (CS/CD) composite membranes were prepared to investigate the influence of β-cyclodextrin on permselectivity and permeate flux of CS composite membranes in the enantiomer separation of tryptophan (Trp) racemate (Wang et al. 2007). Diffusion selectivity, sorption selectivity, and overall permselectivity of the composite membranes toward Trp racemate were investigated in detail. Diffusion selectivity, compared with sorption selectivity, was found to be preponderant in the enantiomer separation process. With the increase of β-cyclodextrin polymer

(β-CDP) content within the CS/CD composite membrane, the permselectivity decreased considerably due to relatively lower complexation selectivity of β-CDP that reduced both the diffusion selectivity and the overall permselectivity. Therefore, the more the β-CDP content within the membrane, the lower the permselectivity of the membrane; on the other hand, permeate flux was found to increase significantly as a result of the facilitated Trp mass transport through β-CDP within the membrane.

The concept of chiral ligand exchange is employed to achieve the chiral resolution of tryptophan (Trp) enantiomers by using chitosan membrane in a sorption resolution mode and copper (II) ion as the complexing ion. Chitosan porous membranes are prepared by freeze-drying method (CS-LT) and sol–gel process at high temperature (CS-HT), respectively, to investigate their sorption resolution characteristics. The proposed CS chiral ligand exchange membranes exhibit good chiral resolution capability. Meanwhile the sorption selectivity of the CS membranes is found to be reversed from L-selectivity at low copper (II) ion concentration to D-selectivity at high copper (II) ion concentration, which is attributable to the stability difference between the copper (II)-L-Trp and copper (II)-D-Trp complexes. Moreover, the CS-HT membrane shows better performance with respect to both sorption selectivity and sorption capability than the CS-LT membrane, which mainly results from its more amorphous structures compared with the more crystalline structures of the CS-LT membrane. The superiority of sorption capability of the CS-HT membrane is also attributable to its larger specific surface area than that of the CS-LT membrane. The results obtained in this study are conducive to the design and fabrication of chiral ligand exchange membranes for enantiomer separation in sorption mode (Wang et al. 2009).

Optical resolution with membranes derived from marine polymers Novel polyion-lipid complexes were prepared from quaterinized chitosan (QCh), which was a derivative of marine (natural) polymer, and three types of anionic amphiphile, such as sodium 1-dodecanesulfate (C12SNa), sodium 1-tetradecanesulfate (C14SNa), and sodium 1-hexadecanesulfate (C16SNa). Those complexes gave durable self-standing membranes. The QCh-lipid complex membranes prepared in the present study showed chiral separation ability; in other words, they selectively transported L-Lys from racemic mixture of Lys adopting a concentration gradient as a driving force for membrane transport. Permselectivity of QCh-C12S membrane toward L-Lys was determined to be 3.31 under the concentration difference of 1.0×10 mol dm. From transport experiments and adsorption studies, it was revealed that the permselectivity was dominantly determined by diffusivity selectivity. It is expected that the present study would open a door to novel materials (Ogata et al. 2010).

The optical resolution of tryptophan using a chitosan functionalized cellulose acetate membrane is investigated in three processes with different driving forces (Zhou et al. 2012). The enantiomeric excesses obtained using the same membrane in concentration gradient, hydraulic pressure and electric field driven processes are 94%, 66% and − 19%, respectively. This reverse in the enantioselectivity in the

electric driven process is also observed in phenylalanine resolution and is mainly due to the orienting force exerted by the electric field and the tryptophan complexes formed with copper ions generated *via* electrolysis. The minimized total energies of the copper complexes and the polymer amorphous cells are calculated with the aid of molecular simulation and the membrane surface morphology is observed in SEM and AFM. Without chitosan and solvent evaporation, the cellulose acetate membrane does not show much enantioselectivity. The necessities of chitosan functionalization and solvent evaporation during membrane preparation, and the effects of operating conditions in chiral resolution tests are also studied in detail.

Nanofiber membranes for chiral separation were prepared from chitin, which is the most abundant natural amino polysaccharide. The membrane showed chiral separation ability by adopting concentration gradient as a driving force for membrane transport. In other words, the chitin nanofiber membrane selectively transported the D-isomer of glutamic acid (Glu), phenylalanine (Phe), and lysine (Lys) from the corresponding racemic amino acid mixtures.

Chitin nanofiber membranes were fabricated *via* electrospray deposition. The nanofiber membrane adsorbed L-isomer from racemic mixture of Glu and Phe in preference to the corresponding antipodes, while D-Lys was preferentially incorporated into the membrane. The adsorption isotherms revealed that all enantiomers studied in the present study were incorporated into the nanofiber membrane without any specific interaction, in other words, those were simply adsorbed. In membrane transport experiments, D-isomers were preferentially transported through the nanofiber membrane. In order to attain higher permselectivity, a multi-stage cascade separation was applied in the present study. As expected, the permselectivity toward D-enantiomer was enhanced exponentially with the increase in number of stages from one to five stages *via* three by applying a multi-stage cascade membrane separation (Shiomi and Yoshikawa 2013).

5.4 Conclusions

In this chapter the relation between the structure and the permeation and separation characteristics of chitin and chitosan derivative membranes in resources, energy, environmental and medical field is discussed.

Chitin and chitosan have many functional groups, and can be easily introduced hydrophilic, hydrophobic groups, and can be functionalized by introducing a special functional group. Chitin and chitosan derivatives which are environmental friendly are applied to the membrane science and technology. In the near the appearance of further excellent membranes from chitin and chitosan is expected.

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Chapter 6
Utility of Chitosan for 3D Printing and Bioprinting

Thomas J. Kean and Maya Thanou

Abstract Traditional prototype development and optimization is a long and costly process. Customization of those products is either very difficult or unfeasible. Healthcare implants are often chosen by the surgeon, much like shoes, for the 'best fit'. In addition to synthetic implant issues, there is a considerable lack of tissue and organs for transplant. When we consider the development and testing of new drugs, many *in vitro* models are poor predictors for drug efficacy. Cell and tissue growth on commonly used plastics, in 2 dimensions, may be part of this issue. Looking at drug delivery, the release and stability are often poorly optimized, with controlled drug delivery and release kinetics often unaddressed. In addition to these healthcare related issues, the world is facing increased pressure for resources due to both population growth and standard of living increases.

3D printing and 3D bioprinting offer potential solutions to these problems. There is no doubt that a new era of manufacturing is upon us; 3D printing has revolutionized the way products are made, developed and customized. Chitosan has captured a small area of these fields at 1.1% of total 3D printing and ~4% of bioprinting publications. The open source movement has made the instrumentation, modeling and hardware control software more accessible, further improving the customizability of products. This will also likely increase the number of studies using chitosan. Within the biomedical arena where chitosan and its derivatives have been used, chitosan has found utility across many cell types, including mesenchymal stromal cells and induced pluripotent stem cells, and in modeling tissues such as bone, cartilage and liver. A 3D printed chitosan-based structure was able to record breathing rate, pulse rate, and finger and bicep flexion due to changes in conductivity when compressed. As a biodegradable polymer, chitosan can be added to the list of low resource and low environmental impact 3D printing materials.

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This chapter focuses on the use of chitosan in 3D printing and bioprinting, highlighting its application in drug delivery and tissue engineering.

Keywords Chitosan 3D printing · Chitosan bioprinting · Tissue engineering · Drug delivery · 3D printing · Bioprinting

Abbreviations

DIY	do-it-yourself
DLP	digital light processing
DMLS	direct metal laser sintering
EBM	electron beam manufacturing
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
FDM	fused deposition modeling
IL	interleukin
iPSCs	induced pluripotent stem cells
LAP	phenyl-2,4,6-trimethylbenzoylphosphinate
MSC	mesenchymal stromal cell
PCL	polycaprolactone
PEG	polyethylene glycol
PLA	polylactic acid
PLGA	poly(lactic-co-glycolic acid)
PLL	poly-L-lysine
SLA	stereolithography
SLS	selective laser sintering
SPIONS	superparamagnetic iron oxide nanoparticles
TCP	tricalcium phosphate
TED	technology, entertainment and design
TNFα	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

6.1 Introduction

The use and manufacture of tools as a uniquely human trait has been debunked (Shumaker et al. 2011). However, the evolution and variety of our tools stands as a hallmark of our success as a species. Increases in population have resulted in pressure for resources such as energy, materials and water. Optimizing production, recycling and distribution reduces the pressure on these resources. As technology advances, new and more complex solutions arise to problems both old and new;

from addressing an organ transplantation shortage to improving water purification, 3D printing could hold an answer.

Three-dimensional printing is the process of additively forming objects using computer controlled hardware. This chapter will give a brief description of the techniques of 3D printing and bioprinting, followed up by a focus on examples that have utilized chitosan.

3D printing has great potential and application across multiple fields both as a method for rapid prototype development and, more recently, as a final product manufacturing technique. 3D printing and bioprinting have benefited from both the open source movement and the customizable fabrication options they offer. They have the potential to reduce pollution and decentralize manufacturing (Lupton 2016; Mohammed et al. 2017); however, failed designs and prints can generate a volume of waste that, without attention, diminishes that improvement. With the 'democratization' of the manufacturing process, copyright and intellectual property issues abound; for an interesting discussion of those issues see Mendoza (2015).

3D printing and 3D bioprinting have a relatively brief history (Fig. 6.1), with the first example of 3D printing demonstrated by Kodama in 1981 (Kodama 1981). Bioprinting, the adaptation of 3D printing methods to print with cells, was pioneered by Wilson and Boland (2003).

Currently (as of 25/10/18) 54 clinical trials of 3D printed materials are listed on clinicaltrials.gov, none of which includes chitosan in their application (ClinicalTrials 2018). In terms of chitosan's capture of the 3D printing and bioprinting arena, it is very low at only 50 published studies in total of 4353 listed on PubMed (1.1%; Fig. 6.2), estimated at 4% of the bioprinting studies (Aljohani et al. 2018). This is

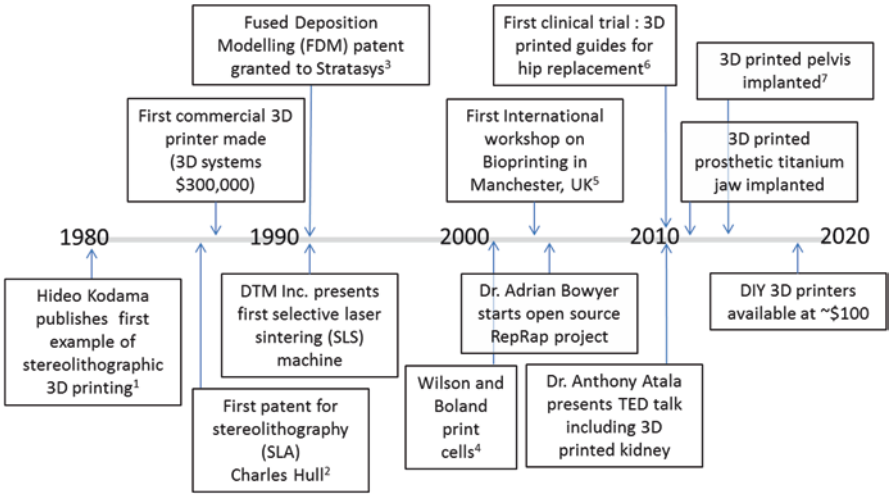


Fig. 6.1 Timeline of 3D printing and bioprinting
Timeline of events in 3D printing and bioprinting. *TED* technology, entertainment and design, *DIY* do-it-yourself, *UK* United Kingdom. [1](Kodama 1981); [2](Hull 1986), [3](Batchelder 1997), [4](Lupton 2016; Wilson and Boland 2003), [5](Mironov et al. 2006), [6](Small et al. 2014), [7](Liang et al. 2017)

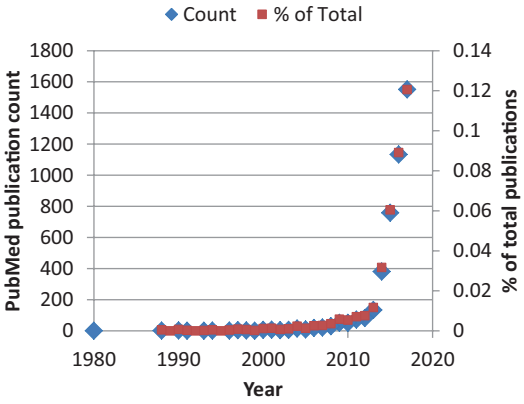


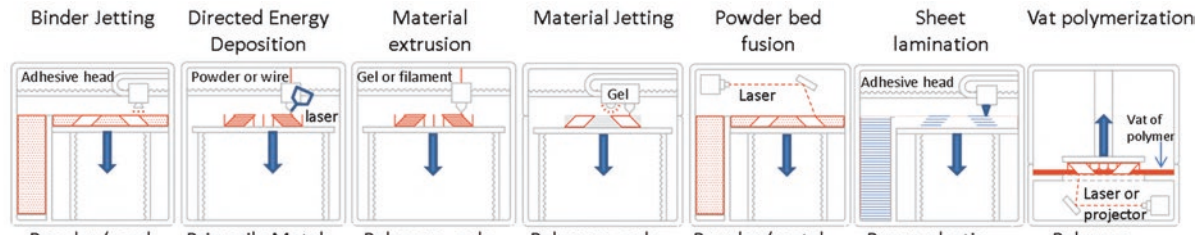
Fig. 6.2 3D Printing publication growth The growth is publications on 3D printing shows an exponential increase in publications indexed by PubMed when queried with the term 3D Printing (PubMed 2018). Blue symbols show number of publications (left y-axis); red symbols show the percent of total publications (right y-axis)

somewhat surprising given its many attractive qualities including: low cost (as a food byproduct), biodegradability, easy modification and biocompatibility (Kean and Thanou 2010), but it is potentially due to the relative novelty of the field. Many 3D printing technologies are still in the innovation phase e.g. 4D printing, whilst some are thought to have reached the peak of inflated expectations e.g. 3D printed drugs, entered the trough of disillusionment e.g. 3D bioprinted human tissue or be climbing the slope of enlightenment to the plateau of productivity e.g. 3D printing of dental devices (Dedehayir and Steinert 2016). While the truth of where the different aspects of 3D printing technology lie on the hype cycle will only be known in hindsight, what is clear is that there is a large scope of applicability and that this is a disruptive technology i.e. one that is fundamentally changing manufacturing and creating new fields of enterprise.

6.1.1 3D Printing Methods

The term 3D printing or additive manufacturing encompasses a variety of methods of fabrication whereby an object is created additively in 3 dimensions. The methods fall into seven areas as defined by the ASTM (2015): binder jetting, directed energy deposition, material extrusion, material jetting, powder bed fusion, sheet lamination and vat polymerization (Fig. 6.3).

The materials that have been utilized in 3D printing are vast and are continuously being added to; metals, ceramics, composites and biomaterials have all been 3D printed. In all of the methods, a digital 3D model is translated to a physical 3D model by computer controlled material deposition in an additive fashion, usually layer by layer. 3D printing has found application in a wide range of areas, from small, intricate jewelry designs to food science (Sun et al. 2015) and house printing (Cowan 2018). In fact, feature resolution of prints is now possible down to the micron/sub-micron level with laser chemical vapor deposition; for review see (Vaezi et al. 2013).



Material examples	Powder (sand, plastic)	Primarily Metals	Polymers, gels	Polymers, gels	Powder (metal, plastic)	Paper, plastic, wood	Polymers, photoactivated
Common terms			FDM, bioplotting	Inkjet, drop on demand	SLS, DMLS, EBM		SLA, DLP
Chitosan use							
3D printing	✓	×	✓	×	×	×	✓
Bioprinting	×	×	✓	✓	×	×	✓

Fig. 6.3 Types and examples of 3D printers and their use in chitosan printing
Many 3D printers print in XY with a movable head then the stage moves down in the Z direction as indicated by the blue arrows. However, there are many examples of 3D printers where the head also moves in the Z direction and/or the stage moves in the XY direction. 3D printers print with a range of materials, bioprinters are usually found in the classes of material extrusion, material jetting and vat polymerization. *FDM* fused deposition modeling, *SLS* selective laser sintering, *DMLS* direct metal laser sintering, *EBM* electron beam manufacturing, *SLA* stereolithography, *DLP* digital light processing. (Reprinted (adapted) with permission from images in articles by Varotsis (2018) at 3D Hubs)

Most areas of 3D printing have been extensively studied. Readers are directed to: Binder jetting (Gokuldoss et al. 2017; Ligon et al. 2017); Directed energy deposition (Martin et al. 2017; Wang et al. 2016); Material extrusion (Ligon et al. 2017); Material jetting (Ligon et al. 2017); Powder bed fusion (Ligon et al. 2017); Sheet lamination (Ligon et al. 2017); Vat polymerization (Ligon et al. 2017; Mondschein et al. 2017; Selimis et al. 2015). For a review of hydrogel characteristics for extrusion based 3D printing, see Kirchmajer et al. (2015).

It is worth noting that, although not included in the ASTM standard list of 3D printing strategies, indirect printing has also found application in chitosan-based methods. Indirect printing first prints the structure in a dissolvable material, infiltrates the desired construct around that material, then the print is dissolved away leaving your product (Do et al. 2015). Another technique that is worth mentioning is that of coating (Sect. 6.4), there are many examples of chitosan coated 3D printed scaffolds; in the majority of these, chitosan has been added to aid in cell attachment and growth.

6.1.2 3D Bioprinting Methods

For the purposes of this chapter, bioprinting techniques are those that include living cells when printing. Many aspects of bioprinting are clearly in the innovation phase, with printing of tissues and organs a challenging goal. When you shift from the realm of printing inert materials to printing living cells, many more factors become necessary to consider. Cells or spheroids (small balls of cells) require biocompatible conditions for printing. Biocompatible conditions must take into account temperature, time to print (nutrient and oxygen requirements), printing solution, mechanical effects and humidity. For a review on current bioinks and their properties see (Gopinathan and Noh 2018). When developing the printing solution, multiple scales (macro, micro, nano) should be taken into account (Stevens and George 2005). On the macro scale, overall strength, shape, handleability, channel or vessel design and potentially (depending on the size of the construct) integration with a perfusion system should be considered. On the micro scale, strand size and orientation, pore size, cell seeding and nutrient availability are important factors. On the nano scale, you have material type, nanotopography, and molecular alignment to be considered. Bioprinting has great potential both in the short term, by providing more relevant cell culture models for drug discovery, and in the long term, as a potential method for organ replacement (Do et al. 2015; Mandrycky et al. 2016). In addition to all the previously mentioned complexities, because it is a living tissue, the fourth dimension of time must also be considered (Vijayavenkataraman et al. 2018). What will the cells do to the scaffold they are printed in/on and how do you direct their ultimate structure? Whilst it can be argued that by anticipating a degree of contracture, through growth factor and mechanical stimuli you can guide the ‘final’ product shape, some have gone a step further and incorporated stimuli responsive biomaterials or cells to enable more precisely controlled 4-dimensional products (Ashammakhi et al. 2018).

6.2 Chitosan 3D Printing

6.2.1 Drug Delivery Applications

Drugs often suffer from several limitations such as poor solubility, burst release profiles and off-target effects such as cytotoxicity. In an interesting study, two-photon crosslinking of methacrylamide chitosan with polyethylene glycol (PEG) super-paramagnetic iron oxide nanoparticles using phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) photoinitiator was used to create screw shaped microswimmers (Fig. 6.4; (Bozuyuk et al. 2018; Yu et al. 2007)). The group was able to control the direction and path of the microswimmers in water in 2 dimensions, with future studies in body fluids planned. These microswimmers were loaded with azide modified doxorubicin using a photocleavable crosslinker, the group then showed light triggered release from the constructs (Bozuyuk et al. 2018). This is an interesting application of 3D printing technology which could allow for specific and controlled release in a spatiotemporal fashion, and it will be interesting to see future feasibility studies. Regardless of whether this application is successful, the group have provided an important piece of information: methacrylated chitosan is 3D printable at a high resolution using two-photon polymerization.

From other studies on PEG-diacrylate, we can see that the crosslinker, LAP, is potentially biocompatible (Fairbanks et al. 2009), meaning that this 2-photon cross-linking system could be used in bioprinting. Another technique that has not been utilized in 3D printing but which deserves mention is that of thermal cross-linking, demonstrated by Bernabé et al. (2005). Ionic cross-links, formed through the interaction of the amine of glucosamine in chitosan with the carboxylic acid of glucuronic acid in pectin, become amide bonds at 120 °C under nitrogen. This has obvious application either during printing or as a post printing modification.

Several other groups have shown the utility of chitosan in drug delivery studies, Yan et al. (2018) complexed carboxymethyl chitosan with Snakegourd root/Astragalus polysaccharide as a potential diabetes treatment. A significant aspect of their study was the effect of the printed fiber orientation on release kinetics. A pH-dependent release was found by Gioumouxouzis et al. (2018), although it did not appear to be an effect of chitosan. Sommer et al. (2017) described 3D printing of concentrated emulsions stabilized by chitosan-silica nanoparticles. Chitosan oligomer (water soluble) was adsorbed onto 22 nm colloidal silica and used to form an emulsion for hydrophobic encapsulation. This chitosan-silica stabilized emulsion has potential in controlled drug release and bioactive scaffolds.

An impressive change in drug release kinetics was achieved by Vorndran et al. (2010), by printing hydroxypropylmethylcellulose or chitosan with brushite into beta-tricalcium phosphate. They were able to change the release profile of vancomycin from a first order burst release, to a zero order continual release profile, over a 3–4 day time period. The primary application of the bioceramic scaffolds made by Vorndran et al. would be in the field of orthopedics, where an implant would be loaded with bone morphogenic protein or vascular endothelial growth factor to encourage bone and vascular ingrowth.

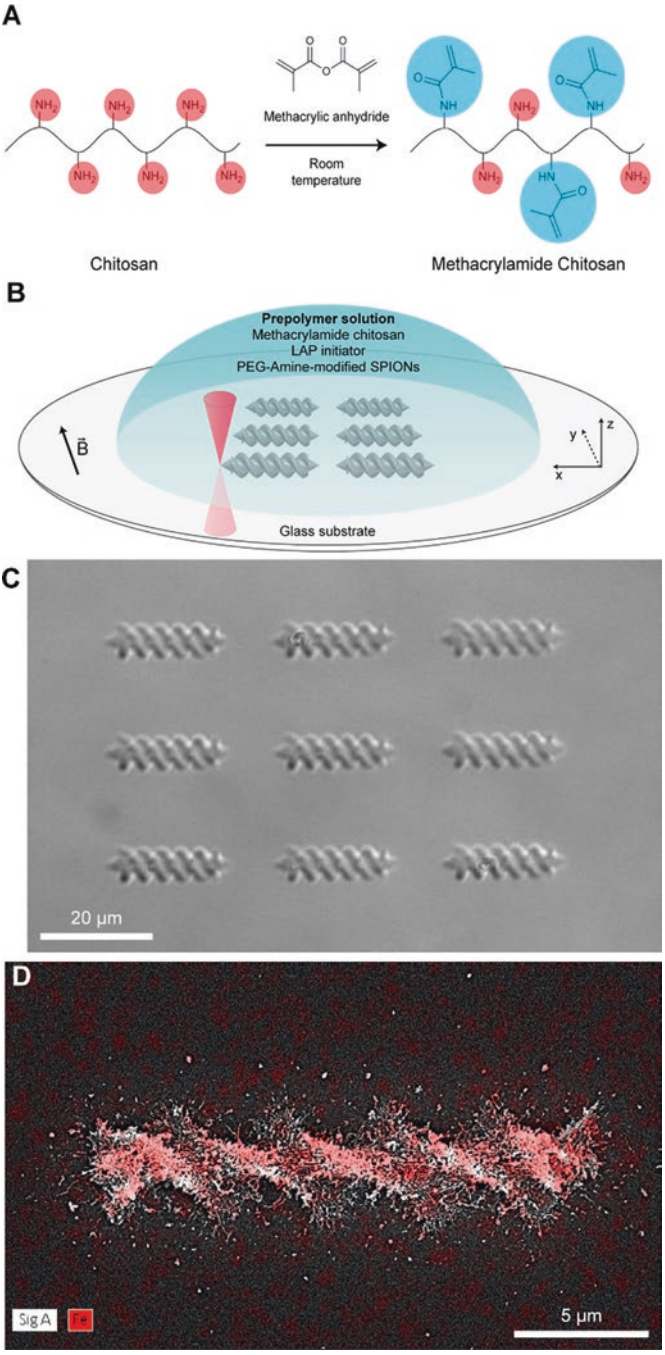


Fig. 6.4 Drug delivering chitosan microswimmer fabrication overview
(**a**) Chitosan was functionalized with methacrylic anhydride to give methacrylamide chitosan. (**b**) Methacrylamide chitosan was reacted with Amine-PEG-SPIONS using LAP photointiator and

In summary, these studies using chitosan have shown its application across a broad range of drug delivery challenges, achieving targetable localization and controlled release profiles.

6.2.2 Conductive 3D Printed Sensors

Conductive 3D printed sensors have many applications including biomimetic prostheses and health monitoring systems. Darabi et al. (2017) modified chitosan with methacrylic anhydride then reacted that with polypyrole. Acrylic acid was polymerized in the presence of chitosan polypyrole and ferric ions, stabilized with N,N″-methylene bis-acrylamide crosslinking resulting in a conductive and self-healing hydrogel. This self-healing hydrogel was able to stretch 1500% and repair in 2 minutes at room temperature. A 3D printed structure was able to record breathing rate, pulse rate, and finger and bicep flexion due to changes in conductivity when compressed. A self-healing stretchable nano-composite chitosan/carbon nanotube sensor was also described by Wu (2018).

6.2.3 Chitosan Scaffolds as a Support for Cell Growth

Cell culture has typically been performed on a 2D plastic surface, this facilitates microscopic observation but is somewhat artificial in its recapitulation of the *in vivo* environment. Culture in a 3D system may provide an improved *in vitro* model. The studies described in this section are distinct from those described later (Sect. 6.3) in that the scaffolds are created first, often post-processed, then cells are added or they are implanted. In Sect. 6.3, cells are printed within the scaffolds. In one of the few studies on printing of unmodified chitosan alone, Wu and collaborators show high (30 μm) resolution prints (Wu 2018; Wu et al. 2017). Chitosan was dissolved in a mix of acetic, lactic and citric acids, and the initial structure is maintained during printing due to evaporation of the acetic acid and high (8–10%) chitosan concentration. Residual acid is evaporated under vacuum, then remaining acid neutralized with sodium hydroxide. The resulting structures maintained their shape characteristics well and had micro/nano-wrinkles due to the drying/rehydration contraction/swelling which may have aided in cell alignment (Fig. 6.4). Several other groups have studied 3D printed chitosan with multiple cell types; these are summarized in Table 6.1.

Fig. 6.4 (continued) 2-photon activation. (**c**) Optical microscopy image of 3D printed microswimmers. (**d**) Energy dispersive X-ray spectroscopy elemental mapping showing the presence of iron atoms (red) in the microswimmers. *LAP* phenyl-2,4,6-trimethylbenzoylphosphinate, *PEG* polyethylene glycol, *SPIONS* superparamagnetic iron oxide nanoparticles. (Reprinted (adapted) with permission from Bozuyuk et al. (2018). Copyright (2018) American Chemical Society)

Table 6.1 3D printed chitosan and its application

Chitosan form	Printing characteristic	Cells/study	References
Solution: chitosan and raffinose in acetic acid	Printed onto a −14 °C bed, further gelled in KOH, 250 μm fibers, 200 μm pores	Keratinocytes and fibroblasts, no increase in healing rate in diabetic wound but potentially better tissue organization	Elviri et al. (2017) and Intini et al. (2018)
Solution: alginate and chitosan	Coaxial extrusion of $CaCl_2$ solution with genipin and EDC crosslinking 130 μm fibers	Hepatocytes, secreted both albumin and urea, CYP34A response to dexamethasone was 50–1000 fold increased over monolayer (functionality testing)	Colosi et al. (2014)
Hydroxybutyl chitosan (temperature responsive gel)	Extrusion of hydroxybutyl chitosan followed by ionic crosslinking with NaCl, then lyophilized; used as a mold	Chondrocytes, grew, no assessment of viability. Higher expression of cartilage marker genes than monolayer culture. Cardiac fibroblasts, aligned with construct	Wang et al. (2018) Tsukamoto et al. (2017)
Coacervation of alginate, xanthan, or κ-carrageenan with chitosan, gelatin, or gelatin methacrylate	κ-carrageenan and gelatin methacrylate found to be best combination	Information on viscosity of solutions and printability (in their system)	Li et al. (2018)
Chitosan, PCL-diacrylate, PEG-diacrylate	SLA with visible light using diphenyl(2,4,6-trimethylbenzoyl) phosphine oxide, dried then rinsed with PBS	Fibroblasts (L929) grew quicker than on tissue culture plastic or PCL/PEG scaffolds when 15% chitosan included	Cheng and Chen (2017)
PLA/chitosan or PLA grafted malic anhydride/chitosan	No printing, assessment of printing materials	Foreskin fibroblasts grew, antibacterial properties shown	Wu (2016)
Chitosan and PEG-diacrylate	SLA with Igracure 819	Low feature resolution, poor MSC engraftment/viability	Morris et al. (2017)
Chitosan with calcium phosphate from egg shell	Extruded as a paste then sintered (burnt out the chitosan)	Good biomechanical properties and produced an *in vitro* osteoinductive surface (MSCs) and in a rabbit model	Dadhich et al. (2016)
Calcium phosphate cement containing chitosan/dextran/BSA or VEGF microparticles	Cement extruded and set in either water or humid atmosphere	Humid atmosphere increased compressive strength, and human dermal microvascular endothelial cell growth	Akkineni et al. (2015)
Chitosan, chitosan/pectin, chitosan/genipin	Solutions extruded into NaOH	Pectin crosslinking doubled stiffness vs. chitosan; osteoblast growth is shown	Liu et al. (2015)

(continued)

Table 6.1 (continued)

Chitosan form	Printing characteristic	Cells/study	References
Gelatin/chitosan	Extrusion of gelatin/chitosan followed by vacuum freeze drying	Adipose derived stem cells spread and grew well	Chen et al. (2014)
Tricalcium phosphate (TCP)/ collagen/ chitosan vs. TCP/ collagen and PLGA	Extruded, washed and ethanol sterilized	Implanted in ovine calvarial defect model, increased bone formation, greater when osteoblasts added	Haberstroh et al. (2010)
Chitosan/gelatin 2.5–7.5%	Extrusion of different percentage composites	Fibroblasts grew well but z fidelity low	Ng et al. (2016)
Chitosan-grafted with L,L-lactide and PEG-diacrylate	SLA: 2 photon crosslinked with Igracure 2959	Rat cortical neurons grew into scaffold	Bardakova et al. (2018)
Collagen/chitosan into gelatin powder	Jet binding method, collagen/chitosan solution printed into gelatin powder	Low resolution, neural stem cells grew well, constructs degraded by 90% in 12 weeks *in vivo*.	Fu et al. (2017)

BSA bovine serum albumin, *VEGF* vascular endothelial growth factor

An important aspect of tissue engineering/bioprinting is the effect of the scaffold on the immune system. This was studied *in vitro* by Almeida et al. (2014), where PLA scaffolds were compared with chitosan scaffolds on mononuclear cytokine production. PLA has higher interleukin (IL) 6, IL12/23 and IL10; chitosan has higher tumour necrosis factor α, an effect that is exacerbated by having a smaller pore size (diagonal geometry). Two significant aspects come out of this study: geometry of the object has an effect, as does surface/chemical composition; however, the correct cytokine stimulation profile is unknown, as cytokine expression and inflammation have a role in repair and regeneration (Fig. 6.5).

In summary, these studies show that 3D culture on more biological substrates offers a representative assessment of cell growth, differentiation and regeneration. It also provides a model to study interactions with the immune system components.

6.3 Chitosan 3D Bioprinting

As a biologically derived scaffold material, chitosan has potential in bioprinting due to its native composition and biocompatibility. In an early study, and one of the longest time in culture models, Yan et al. (2005) extruded gelatin and chitosan with hepatocytes. They increased the rigidity of the construct by crosslinking with tripolyphosphate and a 5s glutaraldehyde crosslinking. Hepatocytes grew for 2-months and produced liver metabolites.

Studies by Gu and collaborators have concentrated on neural cell growth and differentiation; their studies have used an extrusion of a mixture of carboxymethyl-chitosan, agarose and alginate (Gu et al. 2016; Gu et al. 2017; Gu et al. 2018). First,

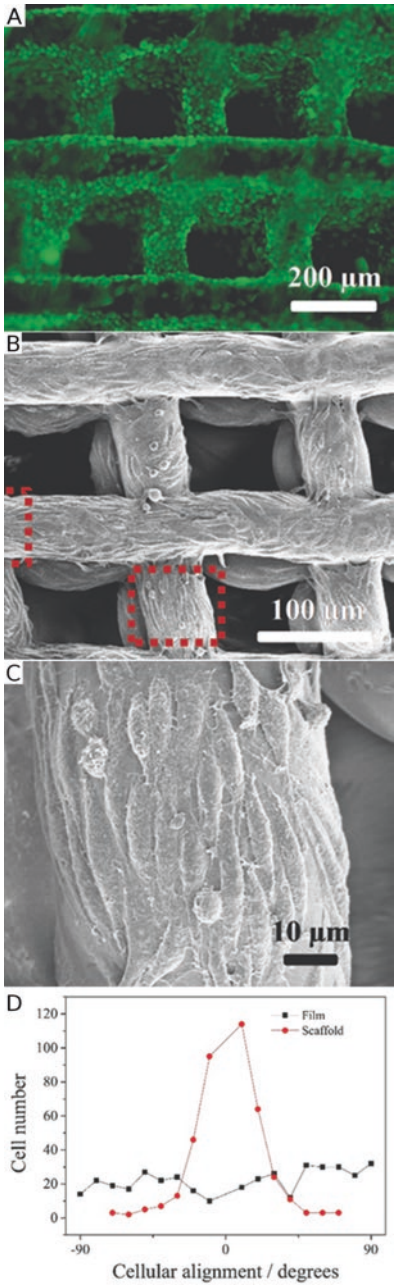


Fig. 6.5 Fluorescence images of L929 fibroblasts on scaffolds (**a**). Scanning electron microscopy images of L929 cells on scaffolds (**b**, **c**), with (**c**) showing a higher magnification image of the red dashed area in B. Quantification of cell alignment to scaffold vs. sheet (**d**). (Adapted from Wu et al. 2017)

they defined the mix of 1.5% alginate 5% carboxymethyl chitosan 5% (a water soluble derivative) as being optimal for print fidelity and human neural stem cell growth (Gu et al. 2016). Constructs were relatively thin, with depth assessment of differentiated cell growth reaching 169 μm. In their later study, they used induced pluripotent stem cells, showed that the scaffold supported embryoid body growth and then directed differentiation along the neural lineage (Gu et al. 2017).

Lee and collaborators developed a chitosan-catechol bioink printing method, catechol was conjugated to chitosan forming a water soluble, mussel-inspired, polymer (Kim et al. 2013; Lee et al. 2018; Ryu et al. 2014). In an extrusion printing method, they found that the addition of vanadyl ions improved the gelation with complexes of serum proteins to form a more stable structure. Fibroblasts (L929) had 90% viability, with printability improving with higher concentrations (20–25%) of fetal bovine serum and chitosan-catechol (10–20 mg/ml).

Using a laser direct write method where cells in alginate were ejected by laser pulse into gelatin/$CaCl_2$, Kingsley et al. (2016) then complexed the microspheres with PLL or chitosan. The alginate core was liquefied by incubation in sodium citrate resulting in encapsulated cell spheroids which could be formed into microstrands. Higher cell viability of a breast cancer cell line (M231) was found with chitosan coating vs. PLL coating. Two cell types, fibroblasts and M231, were printed in a single construct showing discrete localization and interaction between the two. Although this represents a 3D culture, the spheroids or strands were not stacked in this study and the z-axis was approximately 80 μm.

A mixture of chitosan and glycerol phosphate allowed Demirtaş et al. (2017) to produce a chitosan which remained in solution to pH 6.9–7 and had temperature sensitive gelation. A comparison of alginate or chitosan-glycerol phosphate with or without hydroxyapatite printing pre-osteoblasts (MC3T3-E1) was performed. Good cell viability (>90%) was found across all conditions. Osteogenic gene expression supports osteogenesis of MC3T3 cells with chitosan-hydroxyapatite > chitosan > alginate.

Duarte Campos et al. (2015) compared agarose, collagen, agarose-collagen and agarose-chitosan (1%) gel extrusion in air or into perfluorocarbon. Human bone marrow MSCs were suspended in the hydrogels and studied for their adipogenic or osteogenic capacity. Cell viability was high, ~100%, though cell expansion was lowest in the agarose and agarose-chitosan gels. Cells spread most in the collagen gel, particularly under osteogenic conditions. Collagen was also the favored condition for adipogenesis.

These bioprinting studies have shown that chitosan is a viable substrate for iPSCs, MSCs, neurons and other cell lines. They form the basis for future studies creating better *in vitro* models with controlled complexity.

6.4 Chitosan Coating of 3D Prints

6.4.1 Water Purification

Heavy metal and organic contamination of water is an increasing problem, particularly in developing countries. Chitosan has found application in water purification by several groups due to its ability to bind heavy metals, organics and dyes (Lalov et al. 2000; Crini and Badot 2008; Liu et al. 2009; Chen et al. 2013). In one of the only studies found on 3D printing involving chitosan that isn't a biological application, Zhou et al. (2018) took a biomimetic approach. A PLA filter was printed using a fish mouth as a model, this was coated with graphene oxide and chitosan resulting in efficient removal of crystal violet dye.

6.4.2 Biological Applications of Chitosan Coated 3D Prints

These applications of chitosan are similar to those in Sects. 6.2.3 and 6.3, in that chitosan has been applied to the previously fabricated 3D print to enhance cell attachment, growth and function. In an example of indirect 3D printing, Lee et al. (2013) infiltrated a gelatin print with PCL then with chitosan. Firstly a gelatin structure was 3D printed, the gelatin was then dissolved away and the remaining scaffolds either coated with hydroxyapatite or infiltrated by chitosan. The chitosan scaffolds were freeze dried, PCL dissolved with chloroform, followed by hydroxyapatite coating. Mouse bone marrow MSCs growth was improved by apatite coating in both PCL-apatite and Chitosan-apatite.

In a bifunctional approach, Lin et al. (2018) coated an alginate print containing diclofenac with chitosan. Chitosan slowed the release of diclofenac, a non-steroidal anti-inflammatory drug. Osteoblasts (7F2 cell line, spontaneously immortalized from P53 knockout) spread out more on chitosan coated alginate and co-cultured macrophages produced less IL6 and TNFα. They postulated that the chitosan coating protected the cells from macrophage inflammatory signals. The chitosan coated scaffolds also calcified more readily than the alginate alone in osteogenic conditions.

To fabricate chondrogenic scaffolds, Ainola et al. (2016) 3D printed a PCL grid and coated it with electrospun chitosan/PEG followed by glutaraldehyde crosslinking. MSCs seeded on the construct, grown in chondrogenic media, expressed chondrogenic genes but showed weak histological staining.

Induced pluripotent stem cells are a promising cell source for tissue engineering but they require complex and time-intensive cell culture methods. Wong et al. (2018) found that chitosan sheets maintained stem cell markers and increased proliferation. They were then able to use these cells in polyurethane prints. The group did not investigate combination of chitosan into the polyurethane hydrogel print.

Direct writing is an adaptation of electrospinning technology where, instead of spinning onto a stationary surface or spinning drum, solutions or melts are deposited

on a computer controlled stage. In terms of 3D printing, it falls under the material extrusion methods. PCL scaffolds were formed by Wu et al. (2016) using direct write having a fiber width of 21 μm and a total height of 123 μm. Scaffolds were coated with chitosan, decreasing tensile strength and stiffness slightly (~20%) but still having 1.47 MPa tensile strength and 13.93 MPa stiffness (Young's modulus). This strength and stiffness is in the range of articular cartilage, stiffer than soft tissues/organs but softer than bone (Brandl et al. 2007; Currey and Brear 1990; McKee et al. 2011). Similar fibroblast cell growth was seen on both PCL and chitosan coated PCL.

Sousa et al. (2017) deposited chitosan and chondroitin sulphate on nano-patterned templates. This allowed for micron/sub-micron resolution grooves with a depth of 244 nm. Fibroblasts (L929) and myoblasts (C2C12) aligned to the micro-grooves and stimulated myotube formation in growth media conditions.

In another bifunctional approach, extruded PLGA/hydroxyapatite was covalently coated with quaternized chitosan (hydroxypropyltrimethyl ammonium chloride chitosan) by Yang et al. (2018, 2016). They studied bacterial inhibition, MSC growth and bone repair. Significant inhibition of bacterial growth was shown in quaternized chitosan coated samples, including inhibition of a methicillin resistant strain (MRSE287). MSCs adhered, grew and expressed osteogenic markers. Quaternized chitosan coating also encouraged vessel ingrowth into the scaffolds. Bone volume was increased *in vivo* in both a rat femoral defect model and a rabbit femoral condyle defect model.

These combinations of chitosan on a previously formed scaffold have the potential to benefit from the advantages of both systems, e.g. the micropatterning control over scaffold structure achievable with direct write printing and the biocompatibility of chitosan.

6.5 Summary

Chitosan has found broad application, primarily in the field of tissue engineering, within the 3D printing world. Many cell types remain to be studied but iPSCs, MSCs, chondrocytes, fibroblasts, hepatocytes, keratinocytes, myoblasts, neurons and osteoblasts have all been successfully cultured on or in 3D printed chitosan scaffolds. It is good to note that several of these are primary cells rather than cell lines, which are both easier to translate into a tissue engineered therapeutic and more relevant as a drug discovery model. In many cases, a mixture or modification of chitosan has been used, these are summarized in Table 6.2. Further examples of chitosan modifications and mixtures, outside of those used in this 3D printing, are presented in the reviews by Alves and Mano (2008), Rodriguez-Vazquez et al. (2015) and Zargar et al. (2015).

The binding, drug delivery and controlled or triggered release of drugs is likely to be a growth area in 3D printing. Tailored and customizable drug delivery solutions have great potential in both limiting side effects and offering new therapeutic strategies.

Table 6.2 Mixtures and chemical modifications of chitosan

Form	Purpose	References
Mixtures		
Raffinose and chitosan	Raffinose decreases viscosity, improving printability	Elviri et al. (2017) and Intini et al. (2018)
Alginate and chitosan	Alginate cross-links quickly in $CaCl_2$ making stable fibers	Colosi et al. (2014), Gu et al. (2018), Kingsley et al. (2016) and Li et al. (2018)
Xanthan and chitosan	Polyelectrolyte complex gel	Li et al. (2018)
κ-Carrageenan and chitosan	Polyelectrolyte complex gel	Li et al. (2018)
Pectin and chitosan	Polyelectrolyte complex gel	Bernabé et al. (2005) and Liu et al. (2015)
Gelatin and chitosan	Gelatin enables temperature dependent gelling/fiber formation	Chen et al. (2014), Li et al. (2018) and Ng et al. (2016)
Glycerol phosphate and chitosan	Stabilizes chitosan solution to higher pH, temperature sensitive gelation	Demirtaş et al. (2017)
Agarose and chitosan	Agarose enables temperature dependent gelling/fiber formation	Duarte Campos et al. (2015) and Gu et al. (2018)
Chemical modification		
Methacrylamide chitosan	Water soluble derivative and enables cross-linking/ polymerization using photoinitiators	Darabi et al. (2017) and Yu et al. (2007)
L, L-lactide chitosan	2-photon polymerization more effective	Bardakova et al. (2018) and Demina et al. (2017)
Carboxymethyl chitosan	Water soluble derivative	Yang et al. (2018) and Yu et al. (2007)
Hydroxypropyltrimethyl ammonium chloride chitosan	Controlled positive charge density, improved antibacterial properties, water soluble	Tan et al. (2013), Tsukamoto et al. (2017) and Wang et al. (2018)

It is clear that 3D printing and bioprinting are disruptive technologies and, with broader adoption and accessibility, more materials including chitosan and its derivatives will be studied. Chitosan has been used in 4 of the 7 types of 3D printing and could potentially be used in all. New methods of crosslinking (Austero et al. 2012; Donius et al. 2013), modification and equipment will all serve to improve the field and utilization of the useful natural resource of chitosan.

6.6 Future Directions and Conclusions

Chitosan has captured only a small area of its potential applications. We envision its use across more cell types and differentiation protocols to form *in vitro* drug discovery, knowledge generating platforms, and transplantable tissue regenerative solutions.

Layer by layer manufacturing is seen as currently limiting 3D printing; this is due to both the complexities of programming and the current dogma. Accurate imaging/positioning also plays a role to enable a move away from xy then z fabrication. In some arenas, such as 2 photon polymerization, where the photopolymerizable gel can essentially be crosslinked in an xyz direction concurrently because the solution acts as a support, this can also be achieved by printing the bioink into a support solution (Hinton et al. 2015). The ability to modify or print upon a surface easily will also be an important step both in translation to *in vivo* printing/bioprinting and in fabrication of multi-component systems.

Timing is a crucial element in both development and repair. Co-ordinated expression and signaling systems will have to develop along with improved methods for cell and tissue printing. Undoubtedly, 3D printing and bioprinting is already a multidisciplinary endeavor. As we try to create and understand more complex systems, making better *in vitro* models and advancing towards organ transplants, this will require integrated teams from biology, engineering, computing and statistics (at least!). A powerful tool is becoming more accessible and the possibilities are unlimited.

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Chapter 7
The Contribution of D-Glucosamine to Cell Membrane Stability: Mechanisms and Applications in Regenerative Medicine

Yoshihiko Hayashi

Abstract Chitosan is a well-known biomaterial. D-glucosamine, which consists of a natural amino monosaccharide, is the smallest molecular weight of chitosan. D-glucosamine is widely used for relieving the pain associated with osteoarthritis. It has recently been confirmed that D-glucosamine contributes to cell membrane stability in the biomedical field. D-glucosamine may control biological responses and protect both cells and tissues. This chapter briefly focuses the mechanisms of this stability in several important biomedical situations. Positive charged amino groups of D-glucosamine can bind the cell membrane electrically to protect against tissue damage. Wound healing can be accelerated by the application of a D-glucosamine dressing, which promotes cell proliferation and differentiation. D-glucosamine has superoxide/hydroxyl radical scavenging activities and a strong chelating effect on ferrous ions, and enhances the reduced glutathione level to promote activity against intracellular oxidative stress. The early and prompt repair of microleakage through electropores on the cell membrane occurs after electroporation using D-glucosamine. The effects of this stability can also explain the pain relief as D-glucosamine binds to sodium channel to result in a longer open time. Furthermore, specific applications of D-glucosamine are proposed for the regenerative medicine.

Keywords D-glucosamine · Cell membrane stability · Stability mechanisms · Regenerative medicine

7.1 Introduction

D-glucosamine (GlcN), which consists of a natural amino monosaccharide, is the smallest molecular weight of chitosan. It is widely distributed in the connective tissue and cartilage as a component of glycosaminoglycan (Kelly 1998; Dahmer and

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Schiller 2008). It is well known that GlcN which is taken *per oris* as a marine functional food has been used in various countries to relieve the pain of osteoarthritis (Reginster et al. 2001; Gouze et al. 2002; Ma et al. 2002; Cibere et al. 2004; Owens et al. 2004; McAlindon et al. 2004; Zerkak and Dougados 2004; Anderson et al. 2005). Two famous clinical trials using GlcN indicate that for a subset of participants with moderate-to-severe pain, GlcN combined with chondroitin sulfate provided significant relief in comparison to a placebo (Clegg et al. 2006), and that GlcN sulfate was a highly cost-effective alternative therapy in comparison to paracetamol and a placebo for the treatment of patients diagnosed with primary knee osteoarthritis (Scholtissen et al. 2010).

Many other biological activities such as wound healing, anti-inflammatory actions, suppression of adjuvant arthritis, and platelet aggregation have been investigated both *in vitro* and *in vivo* (Hua et al. 2002, 2004, 2005; Matsunaga et al. 2006; Yomogida et al. 2008; Chen et al. 2012; Carames et al. 2013; Wu et al. 2014). Furthermore, an antinociceptive effect of GlcN was recently shown using an electrophysiological method (Kaida et al. 2013, 2014). This chapter describes the mechanisms of the phenomena associated to the contribution of GlcN to cell membrane stability based on the basic research, and proposes specific applications of GlcN in regenerative medicine.

7.2 Mechanisms

7.2.1 Wound Healing

GlcN can physically attach to the cell membrane. Positively charged amino groups of GlcN can bind the cell membrane electrically to protect against tissue damage (Liu et al. 2007; Kawakami et al. 2008; Higuchi et al. 2010). This process may contribute to cell membrane stability, which means that the application of a GlcN dressing would accelerate wound healing due to increased cell proliferation and differentiation (Matsunaga et al. 2006).

GlcN was shown to reduce LPS-mediated NF-κB signaling by reducing IκB phosphorylation, p65 nuclear translocation, and NF-κB reporter activity (Chuang et al. 2013). GlcN also diminished the increase of the TNFα-mediated up-regulation of the MMP-3 mRNA levels on nucleus pulposus cells (Mavrogonatou et al. 2014). These anti-inflammatory effects of GlcN are also arranged and controlled by the membrane stability to accelerate the wound healing processes.

7.2.2 Antioxidant Activity

GlcN has been found to have multiple antioxidant activities, including superoxide/hydroxyl radical scavenging activities, a strong chelating effect on ferrous ions, and protecting macromolecules such as protein, lipid, and deoxyribose from oxidative damage (Xing et al. 2006; Yan et al. 2007; Fang et al. 2007).

Oxidative hemolysis and the lipid/protein peroxidation of erythrocytes induced by a water-soluble free radical initiator, 2,2′-azobis (2-amidinopropane) dihydrochloride were significantly suppressed by GlcN in a time- and dose-dependent manner *in vitro* (Jamialahmadi et al. 2014). GlcN also prevents the decrease in the glutathione level of erythrocytes; this occurs due to the free radical scavenging activity of this compound (Jamialahmadi et al. 2014). Furthermore, GlcN enhanced the reduced glutathione level in oxidatively stressed human chondrocytes; this ability then promotes the potency of its action against intracellular oxidative stress (Mendis et al. 2008). These antioxidant activities may be first brought about by the cell membrane stability of GlcN.

7.2.3 Electroporation

Electroporation is currently used for food and biomass processing (Toepfl et al. 2007; Sack et al. 2010; Martin-Belloso and Sorbrino-Lopez 2011) and as a local cancer treatment (Sersa et al. 2012; Testori et al. 2012). Since all types of cells (human, animal, plant, and microorganism) can be effectively electroporated without the addition of any viral or chemical compounds, electroporation is considered to be both a universal method and a platform technology in the biomedical fields (Miklavic 2012).

The formation of aqueous pores is initiated by the penetration of water molecules into the lipid bilayer of the membrane for gene transfection (Kotnik et al. 2012; Kotnik 2013). The high transfection efficiency after electroporation in GlcN group means that the bioactivity of transfected cells is elevated, and effective gene transportation into nuclei occurs simultaneously (Igawa et al. 2014a, b). The GlcN may bring about the early and prompt repair of microleakage through electropores, thereby contributing to cell membrane stability as an adhesive for polar head groups.

7.2.4 Pain Relief

GlcN reduces the elementary current amplitude and increases the mean channel open time (Marchias and Marty 1980). As GlcN has a weak binding site in the channel itself, its binding to sodium channels, which results in a longer open time, might have an antinociceptive effect (Rogers et al. 2006; Cummins et al. 2007). GlcN inhibits bradikinin- and serotonin- induced nociception (Kaida et al. 2013, 2014). Furthermore, TRPV1 is a cation channel that is activated by various chemical stimulants. The activation of TRPV1 brings painful sensations. Bradykinin can also sensitize TRPV1 via the G protein coupled receptor (Premkumar and Ahern 2000; Vellani et al. 2001). The TRPV1 function is enhanced by 5-HT receptor activation (Sugiura et al. 2004). Thus, GlcN may have an antinociceptive effect by binding to TRPV1. The effects of the cell membrane stability induced by GlcN can well explain these pain relief actions together with the anti-inflammatory effects of GlcN.

7.3 Applications for Regenerative Medicine

7.3.1 Supplement in Cell Culture

The techniques for cell or tissue culture, both *in vitro* and *ex vivo*, are indispensable for regenerative medicine (Yang et al. 2007; Zweigerdt 2009; Kelm and Fussenegger 2010; Ko et al. 2013; Ma et al. 2014; Emmert et al. 2014; Owaki et al. 2014; Leushackke and Barker 2014; Chen et al. 2015; Zhou et al. 2016). The activation of osteoblastic cells is brought about by accelerating the expression of ALP and BMP-2 mRNAs in GlcN-supplemented culture medium (Matsunaga et al. 2006). The expression of signaling-related molecular genes, especially the MAPK pathway, to promote the proliferation of stem cells, is also increased in GlcN-supplemented cell culture (Ganno et al. 2007). Thus, GlcN supplementation of the culture medium is important for allowing the effective proliferation and collection of various stem cells *ex vivo* for application in regenerative medicine.

7.3.2 Dressing Material

GlcN on a liquid or gel type of scaffold can be directly applied as a dressing for use in the field of regenerative medicine for treating defects of soft or hard tissue, such as dental pulp, periodontal tissue, bone, and cartilage. Stem cells may be expected to proliferate and differentiate actively *in vivo* on tissue regeneration. GlcN also accelerates hard tissue formation after a dressing is directly applied to the tissue defect (Matsunaga et al. 2006). Furthermore, GlcN can be used to cover blood clots for a modern pulp revascularization procedure as a regenerative endodontic treatment (Iwaya et al. 2001; Branchs and Trope 2004).

7.3.3 Modifier in Scaffold

In mammals, collagen which accounts for more than one-third of the weight of the protein tissue in the body is the most abundant protein (Patino et al. 2002). Type I collagen is the most popular type in the extracellular matrix (Brodsky and Eikenberry 1982; Gelse et al. 2003). The use of collagen as a biomaterial began in 1881 (Gibson 1990). Many innovations have extended the reach of collagen in the engineering and repair of soft tissue (Lee et al. 2001; Cen et al. 2008; Abraham et al. 2008; Chajra et al. 2008; Shoulders and Raines 2009; Ramshaw et al. 2009; Yannas et al. 2010; Kew et al. 2011; Parenteau-Bareil et al. 2011; Yu et al. 2011; Hayashi et al. 2012; Ferreira et al. 2012; Walters and Stegemann 2014; Chattopadhyay and Raines 2014; Ramshaw 2016; Kuttappan et al. 2016). Biodegradable and weak-antigenic characteristics of collagen are more biocompatible than other natural polymers

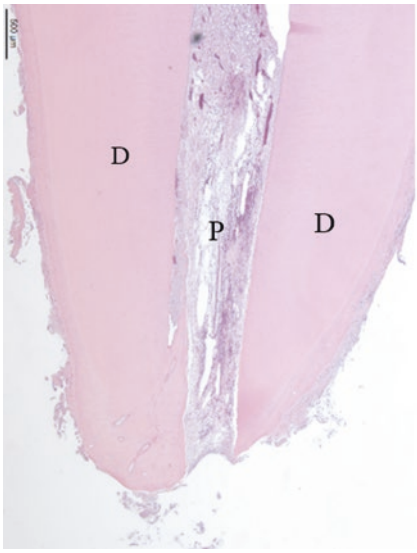

Fig. 7.1 Fish (tilapia) type I atelocollagen solution (FAC) was used as a scaffold. Autologous canine pulp stem cells with granulocyte-colony stimulating factor and FAC were transplanted into the root canal of pulpectomized mandibular canine in beagle dog. Regenerated pulp tissue (P) is occupied over 60% of the root canal space, at 1 month after transplantation. (*D* dentine. Scale: 500 μm)

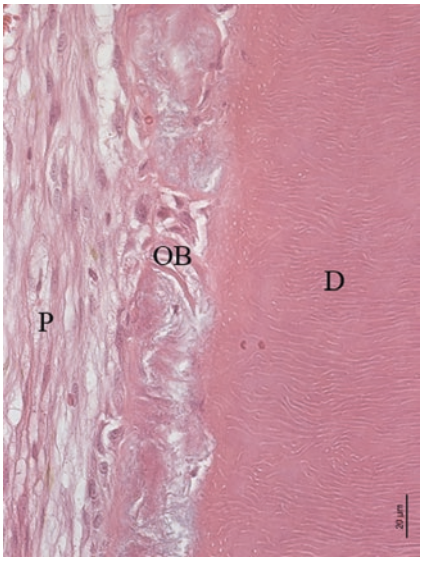

Fig. 7.2 A high magnification view of Fig. 7.1. Regenerated pulp tissue (P) including the vasculature shows no inflammatory reaction. The odontoblastic layer (OB) similar to that in the original pulp is observed at the periphery of the newly formed pulp tissue (P). (*D* dentine. Scale: 20 μm)

(Maeda et al. 1999). Collagen is highly useful as it can be formulated into various types of scaffolds (Chattopadhyay and Raines 2014). More recently, the problem of zoonosis in association with the use of bovine collagen has been overcome, and therefore fish collagen can also now be safely used in biomedical applications (Yamamoto et al. 2014) (Figs. 7.1 and 7.2).

After GlcN powder and the proper concentration of neutralized collagen solution are mixed, the desired structure of the scaffold can be arranged for *in vitro*

(2D- or 3D-culture) and *in vivo* studies. A gel form is easily produced by incubation at 37 °C for 30 min. A film form is produced by air-drying at room temperature. A sponge form is produced by a conventional freeze-drying technique under vacuum evaporation.

7.4 Conclusion

The contribution of GlcN to cell membrane stability was addressed using previous studies on wound healing, antioxidant activity, electroporation, and pain relief. These membrane stability promoting effects are useful in many biomedical fields. Furthermore, GlcN can be applied to promote cell growth and differentiation, while also modifying scaffolds, especially in the field of regenerative medicine.

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Chapter 8
Manufacture Techniques of Chitosan-Based Microcapsules to Enhance Functional Properties of Textiles

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Abstract In recent years, the textile industry has been moving to novel concepts of products, which could deliver to the user, improved performances. Such smart textiles have been proven to have the potential to integrate within a commodity garment advanced feature and functional properties of different kinds. Among those functionalities, considerable interest has been played in functionalizing commodity garments in order to make them positively interact with the human body and therefore being beneficial to the user health. This kind of functionalization generally exploits biopolymers, a class of materials that possess peculiar properties such as biocompatibility and biodegradability that make them suitable for bio-functional textile production. In the context of biopolymer chitosan has been proved to be an excellent potential candidate for this kind of application given its abundant availability and its chemical properties that it positively interacts with biological tissue. Notwithstanding the high potential of chitosan-based technologies in the textile sectors, several issues limit the large-scale production of such innovative garments. In facts the morphologies of chitosan structures should be optimized in order to make them better exploit the biological activity; moreover a suitable process for the

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application of chitosan structures to the textile must be designed. The application process should indeed not only allow an effective and durable fixation of chitosan to textile but also comply with environmental rules concerning pollution emission and utilization of harmful substances.

This chapter reviews the use of microencapsulation technique as an approach to effectively apply chitosan to the textile material while overcoming the significant limitations of finishing processes. The assembly of chitosan macromolecules into microcapsules was proved to boost the biological properties of the polymer thanks to a considerable increase in the surface area available for interactions with the living tissues. Moreover, the incorporation of different active substances into chitosan shells allows the design of multifunctional materials that effectively combine core and shell properties. Based on the kind of substances to be incorporated, several encapsulation processes have been developed. The literature evidences how the proper choices concerning encapsulation technology, chemical formulations, and process parameter allow tuning the properties and the performances of the obtained microcapsules. Furthermore, the microcapsules based finishing process have been reviewed evidencing how the microcapsules morphology can positively interact with textile substrate allowing an improvement in the durability of the treatment. The application of the chitosan shelled microcapsules was proved to be capable of imparting different functionalities to textile substrates opening possibilities for a new generation of garments with improved performances and with the potential of protecting the user from multiple harms. Lastly, a continuous interest was observed in improving the process and formulation design in order to avoid the usage of toxic substances, therefore, complying with an environmentally friendly approach.

Keywords Chitosan · Microencapsulation processes · Textile functionalization · Finishing treatments

8.1 Introduction

The textile industry and research have continuously been evolving over the past 30 years. From the so-called traditional textile product, resulting from conventional processes, whose function was to protect and/or hide the wearer's body, it evolved into more technical products in the 1990s, to become more functional or multifunctional with the development of intelligent textiles, with regard to consumer demands and ecological criteria in the last decade. In this context, smart textiles have gained more and more research and development interests due to their potential functionalities, which bring high added values and increases the market possibilities. In this context, the use of the microencapsulation technologies to manufacture functional coating plays an important role to achieve smart coating textiles (Salaün 2016).

Nevertheless, the use of microencapsulation as a possible tool for the functionalization of textile supports was introduced very late in the process compared to other industrial sectors (Gordon 2001). Nowadays, it is applied for various textile applica-

tions, such as technical, medical or para-medical textiles, cosmetics and textiles for various functional properties such as aesthetic effects, protection, comfort or skin care. Such functional coatings, which provide additional benefits to users without changing the structure and basic characteristics of the product, are becoming necessary in the present highly competitive textile market (Erkan and Sariiski 2004).

The term "microencapsulation" derives from the Greek "mikros" and the Latin "en" and "capsula", small in a little box, respectively. Microencapsulation refers to the formation of polymeric particles entrapping a solid, liquid or gaseous active substance, and exhibiting several types of morphology, ranging from the microscale to nanoscale. This technology aims to immobilize, protect, structure, and release the active principle according to the specific end-use. According to Poncelet, microencapsulation is defined as the entrapment of a compound or system in a dispersed material to its immobilization, its protection, its control of transfer, its structure and its functionalization (Poncelet et al. 2007). Furthermore, the associated terminology to define the obtained particle varies according to the mean diameter. Thus, the term microparticle is used for a range in size between 1 and 1000 μm; macroparticle if it is higher than 1 mm, and for a sub-micronic range the particles are qualified as nanoparticles. The development of the microencapsulation technology is closely related to the progress of many technologies to realize an innovative component for a specific application. Thus, the main characteristics of the microcapsules may be designed as per the application, regarding size and shape, the shell physico-chemical properties, compatibility and permeability. Therefore, microparticles having porous, semi-porous or impermeable shell are used in different applications.

The different synthesis methods proposed in the scientific literature and patents, about 200, describe the formation of microcapsules from 3 stages, i.e., the enclosure of core component, formation of the microparticles, and hardening of it. The microencapsulation processes are divided into three main groups based on the mechanisms formation, i.e., mechanical, chemical and physico-chemical processes. The cost of processing, the desired size, the use of organic solvents for health and environment considerations, the stability of the active principle are the main criteria to select a method rather than another. Furthermore, the polymer-solvent interactions occurring during the microencapsulation process have probably the stronger effect on morphology and properties of the obtained particles. Thus, the solvency of the oil phase affects each step of the microencapsulation process, and the choice of the solvent needs to take in account its properties to induce the polymer precipitation at in the interface during the first stage of the reaction, and also allows the diffusion of the macromolecular chains through this primary shell to induce it growing.

The functional performances of the microcapsules depend on the morphology, the chemical nature and the surface characteristics of the polymeric shell related to the process parameters (Yadav et al. 1990). Microcapsules can have various geometries and structures. Their morphologies depend on the physico-chemical characteristics of the core material, mainly related to solubility parameters, and the mechanism of the shell formation around it. Thus, these particles have either regular or irregular shapes, and on the basis their morphology, the mononuclear or core/shell structure is distinguished from the multinuclear or polynuclear particles, and

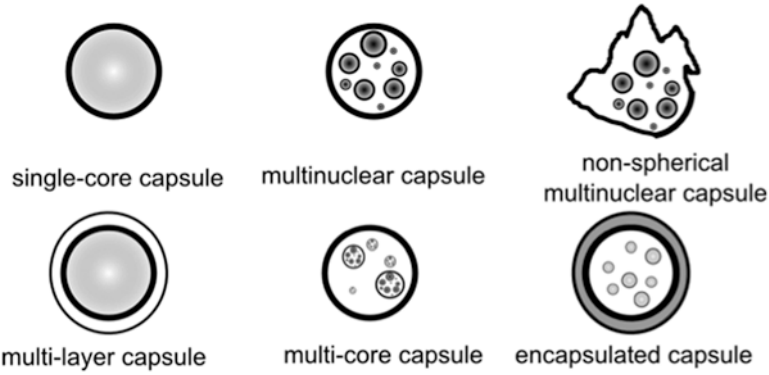

Fig. 8.1 Schematic representation of possible particle structures modified after Kondo (1979). Microparticles have regular or irregular shapes, and mononuclear or multinuclear structures with one or several layered-shell can be obtained

matrix particle or microsphere (Fig. 8.1). The choice of a particular process is determined by the solubility characteristics of the active compound and the shell material for a specific end-use. The bioresourceable and biodegradable polymers, such as chitosan, gelatin, albumin, and alginate have been already used to encapsulate active substance due to their biocompatibility, good release properties, lack of toxicity, film-forming capacity, high mucoadhesivity, and tensile strength (Pedro et al. 2009; Alonso et al. 2010; Garud and Garud 2010).

Chitosan, a linear biopolymer of glucosamine monomers and a small amount of N-acetyl –glucosamine monomers, is obtained by alkaline N-deacetylation of chitin, which is the second most abundant polysaccharide in nature after cellulose (Kasaai 2010). Chitosan is considered to be an attractive biomaterial in the area of microencapsulation technology due to its biocompatible, biodegradable and nontoxic nature (Peng et al. 2010). Chitosan has received significant attention for developing microcapsules, and the main advantages of chitosan-based microcapsules as drug carriers are their controlled release properties and biocompatibility (Rokhade et al. 2007). It is also considered to be a good candidate for wall materials in textile finishing products encapsulation (Alonso et al. 2010). Various methods such as spray drying, phase coacervation have been used for the formation of chitosan microcapsules (Liu et al. 2011); the obtained microcapsules are either single or multilayer depending on microencapsulation method (Pothakamury and Barbosa-Cánovas 1995).

The peculiar chitosan nature, a hydrophilic polymer characterized by the cationic charges in acidic medium, allows designing mild microencapsulation methods in order to obtain micro or nanoparticles. The purpose of this chapter is to analyze the potential use of chitosan to design new textiles substrates and challenges of the chitosan-based textiles products research. The objectives are (i) to provide data on the interest to use this compound to functionalize the textile, (ii) to detail the main applications of chitosan-based textile processing, (iii) to consider the formation of chitosan particles through microencapsulation methods, (iv) to analyze the improvement of the performances of smart coating with these microparticles.

8.2 Chitosan-Based Textile Processing

The whole supply chain of textiles requires research efforts on how to establish the sustainability regarding health safety and cost. The use of chitosan is one of the main opportunities to develop new sustainable industrial practice in fiber production and fabric treatments. Thus, the blended and modified chitosan-based materials extend the availability of various high-end functional textiles with reasonable cost, and then improve the development of the finishing process.

Over the last decade, chitosan has been used in the textile field either under fiber form or in the finishing process as a substitute to conventional polymeric binders. Chitosan fibers are obtained either from spinning processes, i.e., wet spinning, dry spinning, or electrospinning. Chitosan is solubilized in an acid solution before extrusion in an alkaline coagulation bath during the two first methods. The use of chitosan as a binder in the finishing treatment allows protecting the fabric from degradation against the attack of the oxidizer and maintains similar mechanical properties to the untreated bleached fabric. Moreover, it finds some applications in various processes such as a sizing and desizing agent for textile pretreatment (Stegmaier et al. 2008), an auxiliary for dyeing (Ramadan et al. 2011), or a binder for printing (Bahmani et al. 2000).

Cotton fabric has a high shrinkage characteristic, corresponding to an irreversible change in the dimensions, during wetting, washing or drying processes. In most cases, crosslinking agents are used to overcome this phenomenon, and to increase the elastic shrinkage, which is reversible to it. Fragmented chitosan, with an oxidizing agent, allows its diffusion into the textile substrate to create a network with fibers, resulting in the recovery angle increase, and therefore reducing the wrinkle property of fabric, and develop durable press finishing process (Huang et al. 2008).

Chitosan has a high moisture regain value, and therefore it may be applied as an antistatic finishing agent on the textile surface (Abdel-Halim et al. 2010). Therefore, chitosan treated fabrics enable to absorb a very significant amount of water from the surrounding medium, which results in the increase in electrical conductivity following the lower propensity to produce static charges.

Chitosan-treated fabrics and fibers are used for the production of antibacterial fabrics for a working environment such as a hospital, biotechnology research lab, cosmetics, industries, and so on. The charged amino group of chitosan interacts with the cell wall of microbes leading to the degradation of protein and intracellular constituents, and thus their causing cell death (Alonso et al. 2009). These antibacterial properties required significant interactions between chitosan amino groups and cell wall; for this reason, a high concentration of chitosan, which led to the synthesis of nanosized chitosan particles having a higher specific surface area was proposed as a strategy to increase the chitosan-cell interactions. Nevertheless, chitosan is often coupled with other antibacterial compounds such as silver, to fight against large varieties of the microbes (Ali et al. 2011). In most of the cases, the chitosan formulation required the use of a coupling agent, such as glutaraldehyde, butane tetracar-

boxylic acid, citric acid, potassium permanganate, and sodium hypophosphite, to be covalently bonded to the textile fibers, either cellulose or wool.

The adding of graphene particles into a chitosan coating allows developing a cotton fabric as UV blocker (Tian et al. 2015). The modification of the surface fabric state by the incorporation of nanoscale chitosan coating has been found to increase the substrate roughness to provide water repellent properties. Nevertheless, to maintain the shelf life of the hydrophobic behavior, this treatment is usually coupled with silicon and/or fluoride treatment (Ivanova et al. 2013).

Chitosan, being a nitrogen-containing polysaccharide, can provide a char-forming property for use as intumescent additives. Furthermore, it can also act as a blowing agent with the release of nitrogen compounds during its degradation. Nevertheless, to be effective, it needs to be coupled with phosphorous species, such as phytic acid (Laufer et al. 2012), sodium polyphosphate (Charuchinda and Srikulkit 2005), orthophosphoric acid (Abou-Okeil et al. 2007), phosphate-nickel (Hu et al. 2012), melamine phosphate (Leistner et al. 2015), diammonium hydrogen phosphate (El-Tahlawy 2008), etc. The presence of ammonium in the mixture may provide a synergistic effect with phosphate groups for flame retardancy properties (Kandola et al. 1996).

8.3 Chitosan-Based Microencapsulation Methods for Textile Applications

Microencapsulation technology is in growing expansion in the textile field due to the potentiality and versatility in terms of applications. The process allows coating some tiny particles of an active substance with a continuous film of a determined size range. Furthermore, it allows the core materials to be released under controlled conditions enhancing their specific functionalities. The release kinetics mainly depends on the selection of the wall materials, microencapsulation methods, and also the specific end uses. The delivery of the active substance can be achieved by shell permeability changes or degradation from external stimuli, i.e., friction, pressure, temperature, diffusion through the polymer wall, dissolution of the polymer wall coating, or biodegradation.

The advantages of implementing functionalities onto textiles via microencapsulation are as follows:

1. protection of unstable and sensitive agents from the external environment before and during use, i.e., heat, acidity, alkalinity, moisture, or evaporation;
2. controlled or sustained release of the active substance if required through various media;
3. improvement of shelf-life against degradative reactions, i.e., oxidation and dehydration mechanisms;
4. increase in the compatibility between active substances of different nature;

5. increase in the effectiveness of the functionality with the increase of the specific contact surface area,
6. increase in the compatibility of finishes with other chemical processes and the possibility of combined-bath treatments;
7. improvements in solubility, dispersibility, and flowability for better finishing treatments;
8. and convenience in the handling of active substances.

The main suitable microencapsulation methods used for a textile application are based on physical-chemical methods, since they lead to the formation of particles with a mean diameter lower than 40 μm. They can be applied onto the surface substrate by conventional finishing processes. Therefore, they include simple and complex phase coacervation, emulsion precipitation or chemical crosslinking (Table 8.1). Spray drying method may also be used, as a mechanical process, for specific applications or to obtain dried powder.

8.3.1 Coacervation and Precipitation Methods

Phase coacervation is one of the oldest and widely used microencapsulation techniques and can be divided into two groups, i.e., simple coacervation implying the use of one colloidal solute, and complex coacervation, in which the polymeric solution is obtained from the interactions of two oppositely charged colloids. Coacervation corresponds to the separation of a macromolecular solution into two immiscible liquid phases, i.e., a dense coacervate phase and a dilute equilibrium phase. These methods are carried out in four consecutive steps carried out under stirring, i.e., (i) dispersion of the active substance in a solution of a surface-active hydrocolloid; (ii) precipitation of the hydrocolloid onto the dispersed droplets by lowering the solubility of the hydrocolloid, e.g., non solvent, pH change, temperature or electrolyte; (iii) addition of a second hydrocolloid to induce the polymer-polymer complex in the case of complex coacervation; and (iv) stabilization and hardening of the microcapsules by crosslinking agent additions.

8.3.1.1 Simple Coacervation

The simple coacervation is based on the desolvation phenomenon occuring by blowing a chitosan solution into an alkaline precipitation medium, on the addition of a poor solvent to the hydrophilic colloidal solution, resulting in the formation of two phases, i.e., one rich in colloid particles or coacervate, and (ii) the other without coacervate. To induce this, sodium sulfate, alcohol or acetone may add gradually into the solution with continuous stirring.

Chatterjee et al. (2014a) have studied the formation of multilayer microcapsules carried out by phase coacervation method based on ionic interactions between

Table 8.1 Examples of core-shell materials used in chitosan-based microencapsulation for textile applications

Simple coacervation			
Polymer	**Coacervation agent**	**Active substance**	**References**
Chitosan	NaOH	Essential Oil	Souza et al. (2014b), Lam et al. (2013) and Yuen et al. (2012)
		Berberine	
		Miconazole nitrate/Jojoba oil	
Chitosan	SDS	Linseed Oil	Chatterjee et al. (2012, 2014a, b, c)
Chitosan	Sodium tripolyphosphate	Flagrance	Hu et al. (2011)
Chitosan	Glutaraldehyde	Herbal extract	Chandrasekar et al. (2014)
Complex coacervation			
Polymer 1	**Polymer 2**	**Active substance**	**Reference**
Chitosan	Gum Arabic	Dye	Butstraen and Salaün (2014), Wijesirigunawardana and K. Perera (2018) and Sharkawy et al. (2017)
		Lime oil	
		Limonene/vanillin	
		Lemon essential oil	
Chitosan	Silk fibroin	PCM	Deveci and Basal (2009)
Chitosan	Gelatin	Antibacterial compound	Specos et al. (2010), Liu et al. (2013), Prata and Grosso (2015), Chelaru et al. (2015) and Maji and Hussain (2008)
		Citronella oil	
		Patchouli oil	
		Limonene	
		Lemon oil	
		Zanthoxylum limonella oil (Genipin)	
Chitosan	Collagen	Lavender oil	Ocak (2012)
Chitosan	Carboxymethyl cellulose	n-hexadecane	Roy et al. (2018a)
Chitosan	Clay-nano particles	n-eicosane	Genç and Alay Aksoy (2016)
Emulsion – precipitation			
Shell	**Precipitation agent**	**Core substance**	**Reference**
Chitosan	NaOH	Essential oil	Javid et al. (2014)
Emulsion-chemical crosslinking			
Shell	**Shell crosslinker**	**Core substance**	**References**
Chitosan	Glutaraldehyde	Polyurethane photochromic microcapsules	Fan et al. (2015) and Rajendran et al. (2012)
	TPP	Neem extract	

(continued)

Table 8.1 (continued)

Miscellaneous microencapsulation process			
Methods	**Shell hardener**	**Core substance**	**References**
Ionic gelification	NaOH, TPP	Rose fragrance	Hu et al. (2011)
Sonofication	Alginate (layer-by-layer)	Antimicrobial	Antunes et al. (2014)
Spray drying		Vanilin	Yang et al. (2014) and Li et al. (2013)
		Orange oil	
Surfactant-free dispersion copolymerization	PolyNiPAAM-chitosan	–	Kulkarni et al. (2010)

oppositely charged chitosan and sodium monododecyl sulfate (SDS) as wall materials (Chatterjee et al. 2014a). The microencapsulation method started with the preparation of an oil in water emulsion using SDS as an anionic emulsifier. The dilution during microcapsule formation was applied to reduce the repulsion between positively charged microcapsules and free chitosan macromolecules during the microencapsulation process and decrease the viscosity of the microcapsules suspension. The microcapsules formed after 11 alternated additions of chitosan and SDS by phase coacervation process were treated with alkali for drying in a liquid medium to solidify the outermost shell and charge neutralization of amine groups of chitosan on the microcapsules surface. Nevertheless, they observed that this treatment led to the formation of flocculi in the suspension rising to some undesired gel formation. This is the main drawback for commercial use of this slurry in various applications especially for textiles. A small amount of butanol was added in alkali solution to overcome this drawback. The alcohol allows restricting swelling of the outermost shell of microcapsules. After that, the samples were subject to react with trisodium citrate solution for buffering action and ionic cross-linking. Cationic polyamine may attain ordered microcapsule structure under specific salt solutions, and the presence of counter-ion in the solution leads to the formation of ordered microcapsules structure from ionically cross-linked polymers (Rana et al. 2004).

8.3.1.2 Complex Coacervation

When microcapsules were prepared by complex coacervation, the process consists in four consecutive steps. The first step involved the solubilization or dissolution of the biopolymers in aqueous solution. Thus, low concentrated chitosan solution was prepared by dissolving chitosan powder in acetic acid, and left under magnetic stirring for several hours until complete dissolution. The required amount of anionic polymer, Arabic gum or gelatin (Fig. 8.2) was also solubilized in an aqueous medium. In the second step, the two aqueous solutions were mixed together, prior adding of core substance to be emulsified at high shearing rate. In most the cases, the bath temperature is relatively high to decrease the viscosity of the solutions and to favor the obtaining of narrow size distribution and low mean diameter of the

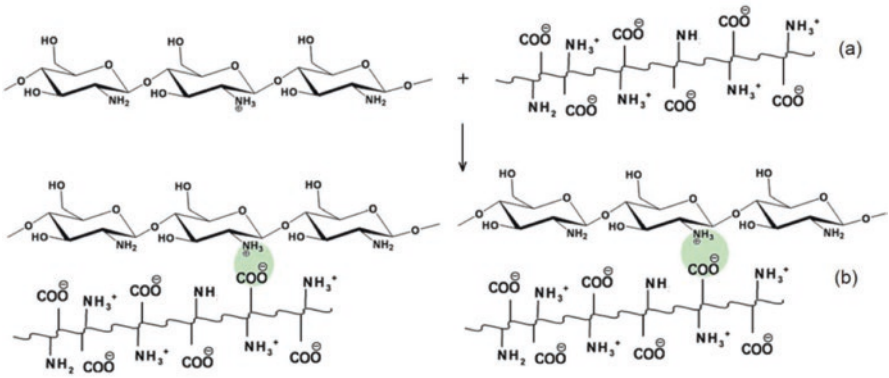

Fig. 8.2 Possible reaction mechanisms: (**a**) chitosan and gelatin microstructure and (**b**) ion exchange in acetic acid and possible reaction mechanism between chitosan and gelatin. (Samimi Gharaie et al. 2018)

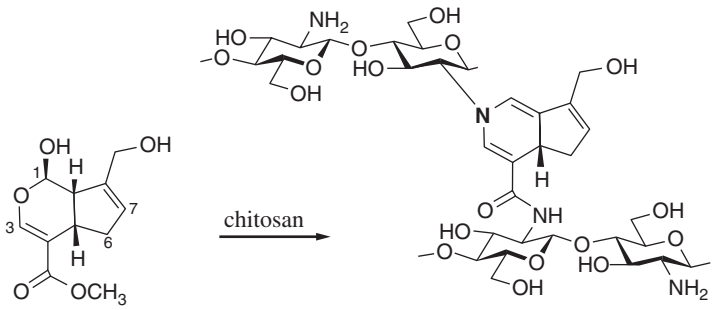

Fig. 8.3 Genipin reacts with chitosan to yield two main crosslinking reactions. On the right, two chitosan chains (represented by their structural units) are crosslinked by one mole of genipin: the formula shows the two newly formed chemical groups, namely the monosubstituted amide and the tertiary amine. (Muzzarelli 2009)

dispersed core substance droplets. The induction of the polymers coacervation, by decreasing the pH with acid at low stirring rate is done in the third step. For example, the maximization of the cationic charges of chitosan is obtained in a pH range between 2.8 and 4, whereas for Arabic gum, the negative charges are found at a pH higher than 2.2. The next step is the gelation of the system by reducing the bath temperature between 5 and 10 °C, and before introducing the cross-linkers such as tannic acid, glutaraldehyde, or genipin (Fig. 8.3).

The synthesis of microcapsules from Pickering emulsion approach is an attractive way to design the particles. This process is based on the preparation of a particle-stabilized emulsion. The diffusion of the particles, solid or coacervates, stabilize the interface against droplet coalescence by reducing the interfacial energy of the system.

Zhang et al. (2018) have studied the ability of functionalized reduced graphene oxide to stabilize chitosan emulsions in w/o Pickering emulsifications, in which toluene is the continuous phase and a chitosan aqueous solution is the discontinuous phase. They observed that the use of functionalized reduced graphene oxide as a stabilizer provides a flexible way to design hydrophilic polymer droplets or microcapsules for controllable drug release behavior. Chitosan-type B gelatin may also be used as Pickering particle to stabilize emulsion (Roy et al. 2018b). The increase of gelatin B amount in the biopolymer ratio, as well as the biopolymer concentration, narrows the particle size distribution, due to the capture of the obtained droplets after the homogenization step in denser emulsions. The formation of chitosan-gelatin complex occurs via non-Coulombic interactions (Kovach et al. 2016). These coacervated particles stabilize the interface, and after coalescence and by introducing a cross-linkers, hardened particles can be obtained.

8.3.2 Water-in-Oil Emulsion and Chemical Cross-Linking

The water-in-oil emulsion followed by chemical cross-linking reaction in one the first method used to the synthesis of chitosan nanoparticles (Fig. 8.4). Chitosan is firstly dispersed in an aqueous acidic phase containing an active substance, emulsified in an organic solvent, such as toluene or paraffin, with a suitable surfactant such

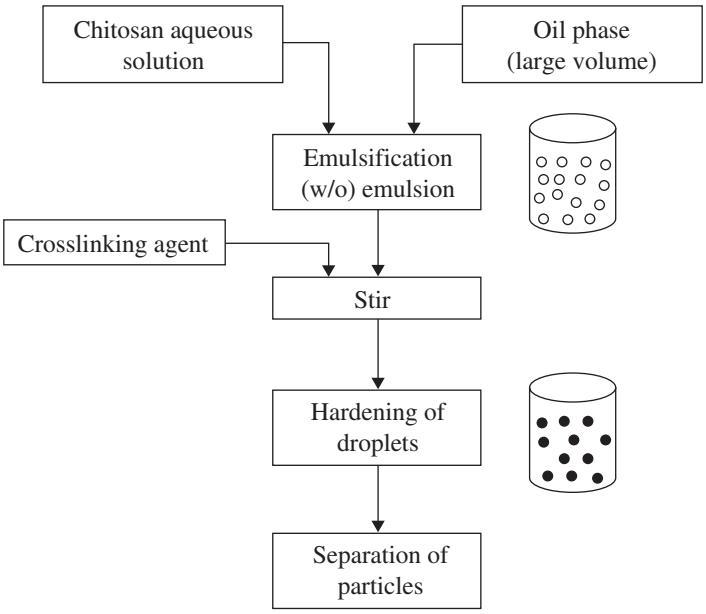

Fig. 8.4 Schematic representation of preparation of chitosan particulate systems by emulsion cross-linking method. (Reprinted with permission from Agnihotri et al. (2004))

Span 80. The emulsion was cooled below 10 °C to induce gelation, and the pH was adjusted to 9–10 with soda. In the last stage, the addition of a cross-linking agent allows the formation of hardening particles. The stirring speed, as well as the amount of cross-linkers, are the main factors affecting the mean diameter and particle size distribution (Agnihotri et al. 2004). One of the main drawbacks of this method is the use of toxic cross-linkers, such as glutaraldehyde (Fig. 8.5). In some cases, the addition of aqueous of methanolic NaOH can also further harden the obtained particles.

Emulsion cross-link method presents few drawbacks, the method is tedious and involves the use of harsh cross-linkers, which can react with the polymer or the active substance. Furthermore, the particle size distribution is sometimes higher than the required one for a textile application.

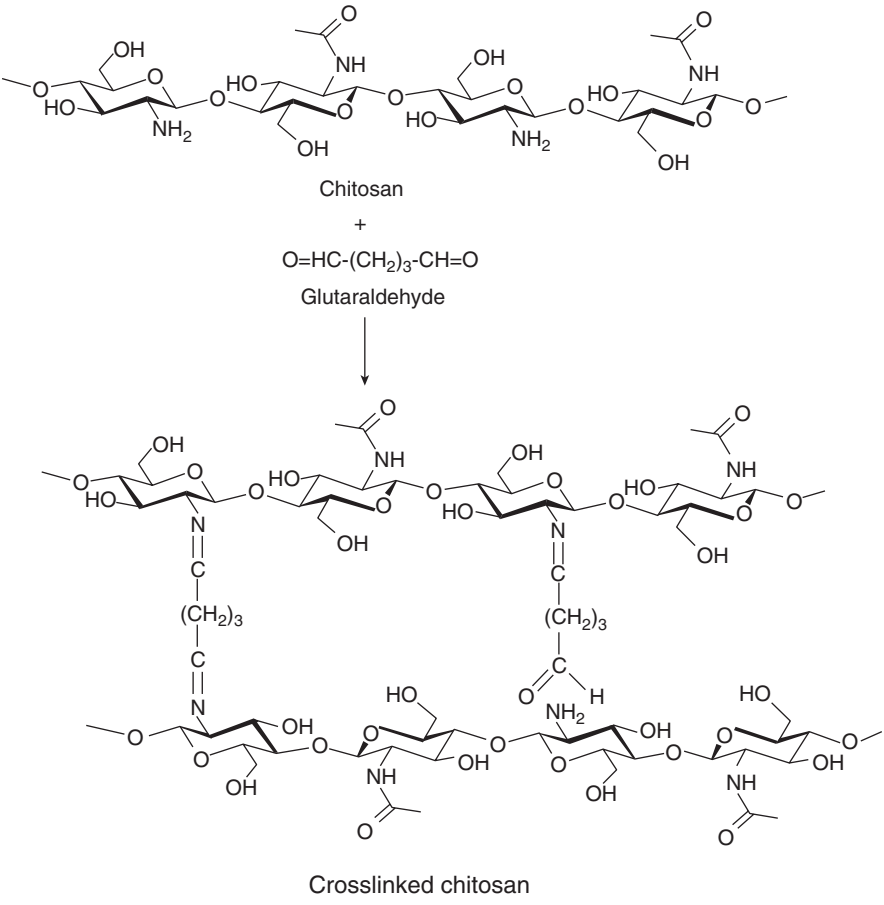

Fig. 8.5 Chitosan molecules reacting with glutaraldehyde to form crosslinked chitosan. (Reprinted with permission from Yang et al. (2004))

8.3.3 *Ionic Cross-Linking Method*

Ionic cross-linking method represents one of the mild ways to synthetize chitosan micro and nanoparticles loaded with an active substance. The formation of the particles is due to the ionic interaction between the positively charge amino groups of chitosan and the anionic charges molecules, such as sodium tripolyphosphate, cyclodextrin derivative, or anionic macromolecules. Thus, this method is based on the ionic attractions of differentially charge macromolecules to synthesize particles. Tripolyphosphate is one of the main common compounds used to induce cross-linking, since it is a multivalent nontoxic molecule, and its tripolyphosphoric groups form gelates through ionic interaction with positively charged amino groups of chitosan. Sodium tripolyphosphate solution is added dropwise to the acidic solution of chitosan, which induces ionic gelation and droplet precipitation (Fig. 8.6). The concentration of chitosan, ratio of chitosan/sodium tripolyphosphate, ionic strength, shear strength, stirring time and pH of the solution are the main parameters influencing the kinetics and the gel formation step. The particle size as well as the surface charge density may be designed or controlled to modify the release behavior and the functionality of the microcapsules.

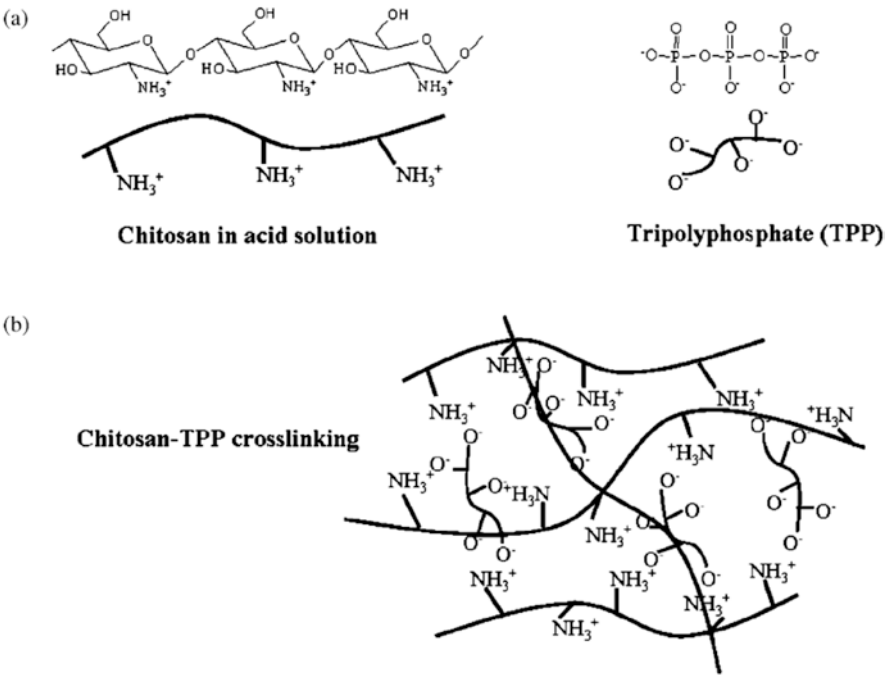

Fig. 8.6 (**a**) Molecular structure of chitosan in acid solution and that of tripolyphosphate (TPP). (**b**) Ionic crosslinking between chitosan and TPP. (Reprinted with permission from Hsieh et al. (2008))

8.3.4 Miscellaneous Microencapsulation Process

Chitosan miscellaneous microencapsulation processes are most of time designed for particular applications, such as a double walled formation, the formation of microspheres, or nanoparticles.

8.4 Textiles Functionalization and Properties

Textile fabrics can be functionalized with chitosan microcapsules by using one of the following finishing treatment (Yip and Luk 2016):

1. prior padding the fabric, it is immersed into the microcapsule suspension followed by curing for fixation;
2. exhaustion method in which the fabric is soaked in the microcapsule suspension for a given time under controlled, the exhaustion process is usually followed by curing for fixation;
3. spraying the microcapsules onto the fabric followed by fixation and/or curing;
4. screen printing microcapsules with an appropriate binder and thickener onto the fabric followed by curing for fixation;
5. and embedding microcapsules onto fabric that has undergone surface modification, such as via atmospheric pressure plasma by using one of the techniques listed in (1) to (4), followed by thermal fixation with a fixing agent that contains a monomeric or oligomeric cross-linker.

8.4.1 Textiles Finishing Treatments

The step of encapsulation allows manufacturing textiles containing microcapsules by various ways to fix the microcapsules within the fiber structure permanently, to embed them into a binder, to mix them into foam or to graft them according to the expected end-use and the microcapsules shell properties. The microcapsules should have some suitable characteristics to be used in the textile field, i.e., a narrow size distribution, a core to shell ratio with a core content as high as possible; stability to mechanical action and high thermal and chemical properties; and also adequate compatibility or affinity with the textile substrate and the binder used. The choice of the textile finishing process to functionalize the fabrics needs to take into account (i) the characteristics of the textile in terms of chemical nature, fiber type, construction design, (ii) the durability of the microcapsules in regards to their effectiveness, (iii) the availability of the machinery, (iv) the cost to benefits ratio, and (v) the environmental considerations and legislation as well as compatibility with other finishes.

Chitosan microcapsules can be applied by conventional finishing techniques or during the rinse cycle of a washing machine on any fabric (woven, nonwoven, knit-

Table 8.2 Examples textiles finishing treatments for chitosan based microcapsules

Application	Incorporated substance	Textile material	Finishing method	References
Antimicrobial	Silver	Polyester	Bath exhaustion	Ali et al. (2011)
Antimicrobial/ antioxidant	Iodine	Viscose	Padding	Zemljič et al. (2017)
Antimicrobial	Pyrazole	Cotton	Pad dry curing	Nada et al. (2018)
Antimicrobial	Berberine	Cotton	Spraying	Lam et al. (2013)
UV-protection/ antimicrobial	Zinc oxyde	Cotton	Pad dry curing	AbdElhady (2012)
UV-protection/ antimicrobial	Zinc oxyde	Cotton	Synthesis of nanoparticles onto fabrics	Perelshtein et al. (2013)
Antimicrobial/ insect repellent	Limonene oil	Cellulose	Padding	Souza et al. (2014b)
Antimicrobial/ insect repellent	N,N-Diethyl-3-methylbenzamide	Cotton	Spraying	Fei and Xin (2007)
Antimicrobial/ insect repellent	Dodecyltrimethylammonium chloride	Wool	Pad dry curing	Hassan and Sunderland (2015)
Cosmetic	Rose fragrance	Cotton	Dipping	Hu et al. (2012)
Cosmetic	Vanillin	Cotton	Chemical grafting	Yang et al. (2014)
Cosmetic	Aromas	Cotton	Chemical grafting	Sharkawy et al. (2017)
Medical	Clindamycin 2-phosphate	Viscose	Padding	Ristić et al. (2016)
Medical	Gallic acid	Cotton	Padding	Hui et al. (2013)
Antimicrobial/ thermal regulation	Silver zeolites+preformed phase change material	Cotton	Pad dry curing, citric acid as cross-linker	Scacchetti et al. (2018)

ted or garments) regardless of its nature (natural, synthetic) (Table 8.2). On the other hand, one of the challenges of the textile research is to ensure the durability of the functional properties of the microcapsules treatment with repeated used, when in some applications they may be damaged during washing cycles (at least 20 washing cycles), ironing or tumble drying.

Impregnation is one of the more appropriate finishing methods to embed microcapsules onto the textile surface due to a lack of affinity between the microcapsules and the textile substrate (Monllor et al. 2007). In most of time, microcapsules solutions are mixed with a dispersant to promote their diffusion through the textile material, followed by the addition of a crosslinking agent in order to bind them to the

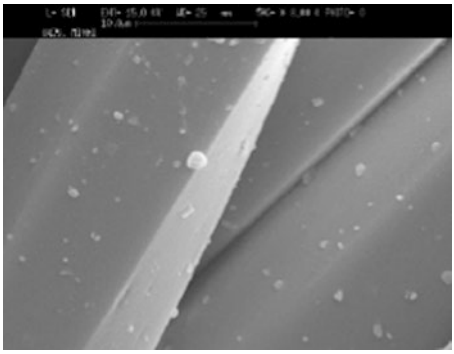

Fig. 8.7 SEM micrographs of polyester fabrics containing microcapsules. Microcapsules are physically linked onto the fiber surface after heating treatment

substrate. In an exhausting process, the fabrics or garments are introduced in a dyeing apparatus with water. A formulation containing the microcapsule dispersion, the polymeric binder, the auxiliaries and acids/bases for pH adjustment, is then introduced in the apparatus at defined times, speeds, temperature, and pH to allow the fixation onto the textiles. In a padding process, the fabric goes through the finishing bath containing the microcapsules, a softener, wetting agents and a binder. After emerging from the bath, the fabric is squeezed by a pair of mangles or rubber rolls at a constant pressure to reach a determined wet pick-up level. The presence of the rolls allows ensuring the contact between the microcapsules and the fibers (Fig. 8.7). Thermal treatment with hot air in an oven is applied to remove water and to cure the resin or binder, and therefore to induce microcapsules adhesion on the fiber surface.

Ionic bonding is another solution to fix charged capsules, such as chitosan, onto the fiber having surface potential, such as polyamide in acidic conditions (Chatterjee et al. 2014a). The capsules are synthesized to have cationic or anionic functional groups on the external surface of the membrane, and therefore imparting affinity and strong ionic bonds between microcapsules and fibers during bath exhaustion treatment without binder. In this case, also, one advantage is that the method may be carried out straightforwardly. The major drawback is the low resistance to wetting since, after a few washes, the capsules disappear from the surface of the fibers.

In some cases, if the function of the microcapsules is to control the release behavior with a porous or semi-porous shell, the use of binder creates a three-dimensionally linked network and is a hindrance of the active substance release. Furthermore, the lack of strong chemical bonding between the capsules and textiles results in poor washing durability and poor air permeability. Even if padding is a simple way to functionalize textile, the applied pressure may break the microcapsules. Chemical grafting represents an efficiency finishing method to overcome these various drawbacks, and therefore to covalently linked the microcapsules onto textile fibers by using polyfunctional cross-linking reagents (Fig. 8.8).

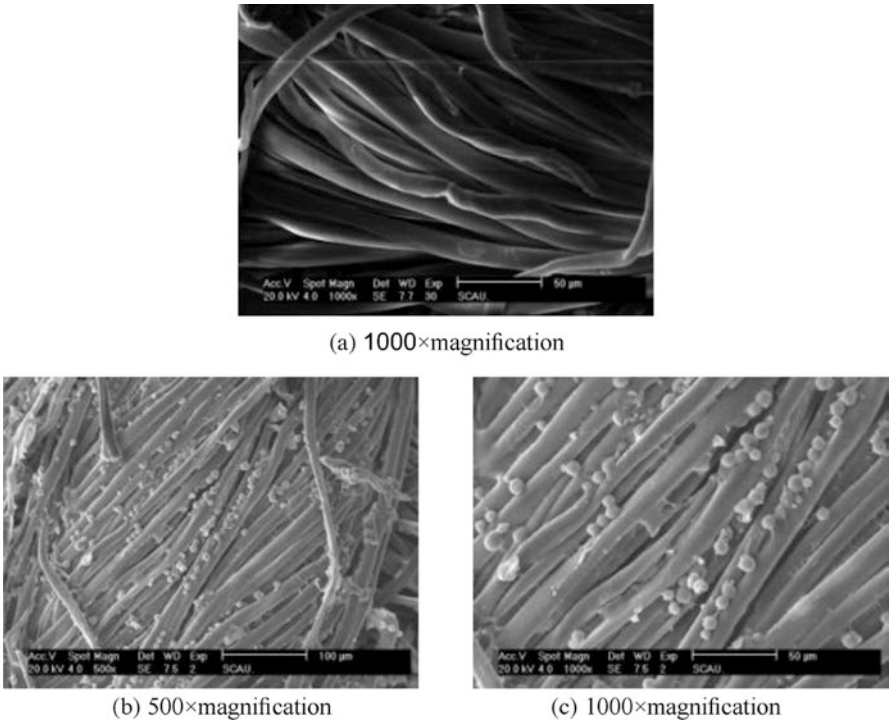

Fig. 8.8 SEM photographs of cotton fabrics before (**a**) and after being treated (**b** and **c**) with chitosan–gelatin microcapsules. (Reprinted with permission from Liu et al. (2013))

8.4.2 Smart End-Uses of Textile Substrates Containing Microcapsules

8.4.2.1 Improving Textile Performances

The research in the field of textile technology has been focusing over the last decades toward the design of garments which can deliver to the user better performances regarding wearability and comfort while imparting functional properties of different kinds (Oliveira and Cunha 2019). In the context of improving the performance of textile materials, microencapsulation was revealed to be a practical approach to combine functional substances with commodity fabrics. In facts the encapsulation process showed the capability of improving the effect of functional substance while easing the binding to the fabric, leading to better performance and durability of the treatments (Salaün 2016). Chitosan represented a very advantageous polymeric shell for the preparation of capsules for functional textile finishing; its natural biodegradability and biocompatibility have made it suitable for encapsulation of bioactive ingredients while its positive surface charge lead to a better affinity for many textile substrates especially the cellulosic ones (Roy et al. 2017).

Furthermore, it showed a natural antimicrobial activity that can be combined with the peculiar properties of the core substance to impart multiple functionalities to the textiles (Konuklu and Paksoy 2015). Scientific literature evidenced how chitosan-based particles have been intensely studied to improve the performances of garments, in particular they found application in the production of medical and cosmetic textiles, moreover significant interest is arising in using chitosan to encapsulate phase change materials as thermal storage devices to improve the comfort of the garment user (Hu et al. 2011; Ristić et al. 2016; Scacchetti et al. 2018).

Cosmeto-textile is a textile material that incorporates particular substances or preparations intended to be released on the outermost layers of skin of the human skin; it holds properties such as cleaning, perfume, modification of appearance, protection, maintenance or repair of body odor. The preparation of cosmeto-textiles has mostly exploited microencapsulation technique in order to avoid the burst release of the active substance and to effectively bind the system to the textile making more effective over longer usage times (Mathis and Mehling 2011).

In the work of Hu et al. (2011), rose fragrance was incorporated into chitosan nanoparticles and applied to cotton fabrics. The synthesized fragrance was incorporated into chitosan nanoparticles by ionic gelation method and attached to the fabrics by dipping them into the nanosuspension under vacuum for 2 h. Release test was conducted by leaving the fabrics in an oven at 70 °C so that the fragrance could be released, at given time interval the fabrics were removed from the oven, and the residual fragrance was extracted in ethanol, the amount of extracted fragrance was analyzed by UV-visible spectroscopy. It was proved that the encapsulated fragrance was retained on the textile while in case of direct application onto the textile substrate all the fragrance vaporized in 10 h.

Yang et al. (2014) incorporated vanillin in chitosan microparticles by spray drying attached the products onto cotton fabrics by crosslinking reaction carried out with citric acid, the crosslinking reaction was carried out using sodium hypophosphite as a catalyst at a temperature of 160 °C. The release test was conducted on the textile for 2 h at different temperature and relative humidity, after the given time the vanillin was extracted from the textile and quantified by gas chromatography. Washing fastness test was conducted in parallel on the textile microcapsule system and on a control sample obtained by directly applying vanillin onto the cotton fabrics. The release test evidenced that the amount of vanillin released is proportional to temperature and relative humidity; the encapsulation process allowed vanillin to be kept onto the cotton fabric for ten washing cycles more than respect to the free vanillin. The author interpreted the data by mean of density functional theory calculation and ascribed the encapsulation and release behavior to the inclusion complexes formed between chitosan and vanillin.

Given the promising results regarding performances some researchers focused on upscaling the production of cosmeto textiles; in doing that it is of crucial importance to properly select the chemical substances involved in the production to avoid harmful and reactants. Therefore, several studies were devoted to the green production of cosmeto textiles in the vision of the industrial process.

The procedure proposed by Alonso et al. (2010) significantly reduced the environmental impact of the process by substituting the chemical grafting with UV curing. Essential oils were loaded in chitosan microcapsules by oil in water emulsion technique, after that, the textile sample was UV irradiated, dipped in the capsules suspension and heated to allow the cross-linking by the esterification reaction.

An in-depth study of the green production of cosmeto textiles was conducted by Sharkawy et al. (2017), which proposed alternatives to toxic cross-linkers and employed both in the capsules production and grafting to fabrics. The main idea of the work was to substitute aldehyde based substances such as glutaraldehyde and melamine formaldehyde with polycarboxylic acids such as tannic and citric acid. The particle preparation was performed by complex coacervation of chitosan and Arabic gum by using tannic acid as a hardener, the process allowed to load vanillin and limonene successfully. The grafting was performed by using citric acid as cross-linker, and the esterification reaction was conducted by employing sodium phosphate monobasic monohydrate as a catalyst in mild temperature conditions (50 °C) after the bath the fabric underwent thermofixing at 90 °C.

The work showed the effectiveness of the green processing since the properties of the cosmeto-textile was not compromised and it represents a good point toward the industrial production of cosmeto textiles.

Similarly to cosmeto-textiles also medical textiles are materials that incorporate bioactive substances, however, while cosmeto-textiles incorporate substances for aesthetic purposes medical textiles aim to have a specific therapeutic target (Shah and Halacheva 2016; Massella et al. 2017). They range of application can be both topical and systemic, in the first case the therapeutic target is the outer skin layers, for example, textile materials have been widely exploited as wound healing devices to treat burns or skin ulcers (Mostafalu et al. 2017). On the other hand, systemic action deals with substances that are released through the skin to reach blood circulation and there exploit therapeutic effect; typical examples of such devices are transdermal patches (Mihailiasa et al. 2016). The first commercialized examples of these devices were based on polymeric films that acted as drug reservoir and could be applied to the patient skin for several days; this however caused skin irritation due to the lack of breathability of the polymeric films. Therefore, textile-based transdermal release system has been gaining significant interest in the recent years. The significant advantage of such materials consisted of the capability of exploiting the high surface area of the skin (the largest organ of the human body) as administration route while making the drug delivery device wearable, therefore no further administration was required by the patient allowing him to be easily compliant to the therapy. Medical textiles have been usually produced by combining commodity fabrics with drug carriers with suitable for topical or transdermal release (Rubio et al. 2010). Such carriers include liposomes, cyclodextrins, polymeric micro and nanoparticles (Martí et al. 2012; Mihailiasa et al. 2016). The already mentioned properties of chitosan made it suitable for dermatological applications, not only for its antimicrobial activity and its ability to incorporate drug (Donalisio et al. 2018). In facts chitosan tends to be degraded in mildly acidic environments, given the normal skin pH to be of about 5.5 the polymer can slowly degrade in the skin environ-

ment thus effectively releasing the drug. Furthermore, the positive surface charge of chitosan eases its ability to cross the skin barrier, which can lead to more effective transdermal release (Khadjavi et al. 2015). Several chitosan-shelled drug carriers have been studied evidencing is good compatibility with skin tissue and no cytotoxicity against skin cells (Argenziano et al. 2017).

In the work of Ristić et al. (2016), clindamycin 2-phosphate was loaded into chitosan nanoparticles by ionic gelation method. The nanoparticles were then used to functionalize viscose fibers by dipping, padding and oven drying. The effectiveness of functionalization was assessed by acid dye adsorption technique. Given the application of the viscose fibers as vaginal tampons, they were tested for antimicrobial activity against Candida Albicans and tested for controlled release in neutral pH at which symptomatic of infection occurring. The study reported the successful loading of the drug in the nanosized carriers and is adsorption on the fiber surface. Furthermore, an excellent antimicrobial efficiency was observed, while the release kinetic was quite fast with a not high delivery of the drug. This can be attributed to fast diffusion of the hydrophilic drug and reduced erosion of the polymer matrix at the pH at which the release test was conducted.

Hui et al. (2013) developed bio-functional textile for the treatment of atopic dermatitis by applying chitosan-sodium alginate microparticles loaded with gallic acid onto cotton fabrics. The gallic acid was extracted from a mixture of Chinese traditional herbs and incorporated in the polymers by emulsion cross-linking process. The application on the cotton fabrics was performed by dipping, padding and oven curing, and a resin binder was employed to enhance the fixation of particles of the textile surface. The drug release test was conducted at pH = 5.5, and the cytotoxicity was assessed on skin keratinocytes cell cultures both by MTT cell viability and LDH membrane integrity tests. The obtained particles displayed a controlled release over 72 h with the release of 90% of drug loaded; this is in accordance with the solubilization of chitosan in acidic pH, which allows a total release of the loaded drug. The cell cultures showed that the biofunctional textile is nontoxic to human skin.

The great potential of the usage of chitosan as material for dermatological applications and the interest in the production of bio-functional textiles makes the application of chitosan based nanocarriers to fabrics a topic of great interest for future research.

One of the recent advancements in textile technologies consisted in the production of garments with improved thermal regulation properties (Dotti et al. 2016). Providing thermal regulation to the human body is the primary objective of garments and nowadays thank innovative finishing it is possible to improve thermal comfort to the user with significant benefits on his performances and health (Mantegazza et al. 2018). Such technologies are of significant interest in developing sportwears for extreme environments. Microencapsulation has been exploited in this sense with the aim of incorporating in the microcapsules the phase changing materials. They are substances of various natures, which can undergo a phase change (usually fusion) in a range of temperature close to the human skin one (35–38 °C).

Moreover, they usually present high enthalpies of phase change (Alay Aksoy et al. 2016). These materials can, therefore, absorb thermal energy from the human body in the form of latent heat, then in case of external temperature change the inverse phase transition occurs and the stored latent heat is released toward the body, therefore phase change materials can contribute to thermal comfort by avoiding heat dissipation if applied to textiles (Yang et al. 2018). Commonly used phase change materials for textiles applications include fatty acids and paraffins; therefore the proper encapsulation in a polymeric shell that can effectively be bound to the fabric is of crucial importance in the scope of imparting fastness and effectiveness to the phase change materials finishing. Recent research has inquired the possibility of using chitosan as polymer shell to entrap phase change materials, the possibility of combining on the same garment the heat management of phase change materials together with the antimicrobial effect of chitosan (Konuklu and Paksoy 2015; Paulo et al. 2018).

Deveci and Basal (2009) studied the encapsulation of n-eicosane by complex coacervation of chitosan with silk fibroin using glutaraldehyde as cross-linker. The study focused on the particles preparation rather than on the testing of system performances. The factorial design of the experiment evidenced how the relative amounts of fibroin and chitosan and silk fibroin are the main factors in determining the final size and encapsulation efficiency of the microcapsules. In the following study, the authors focused on the characterization of the microcapsules, with a particular focus on studying the phase change behavior by DSC analysis (Basal et al. 2011). The enthalpy of melting of the encapsulated PCM was proportional to the quantity of encapsulated n-eicosane with no significant shift in the temperature of fusion which found at 37 °C for the micro PCM system. The authors concluded that the produced microcapsules were suitable as potential heat storage materials for textile application.

Chaiyasat (2018) prepared octadecanone loaded PMMA by exploiting microsuspension iodine transfer polymerization using chitosan as the stabilizer with the aim of preparing a multifunctional thermoregulating and antimicrobial material. The DSC analysis evidenced how the melting enthalpy of the encapsulated octadecanone does not change respect to bulk material, mainly due to hydrophilic nature of the polymer shells which can undergo phase separation during the heating cycle, such results showed the suitability of microcapsules as heat storage system. The testing of antibacterial properties, however, showed scarce inhibition of bacterial growth, attributed to the scarce availability of surface amino groups. The overall system needs to be optimized before being applied to textiles.

An example of a combination of chitosan and PCMs applied to textile substrates is reported by Scacchetti et al. who produced chitosan-silver zeolites microparticles by ionic gelation procedure and applied to cotton fibers together with preformed PCM microcapsules (Scacchetti et al. 2018). The finishing consisted in pad-dry cure process, which employed citric acid as cross-linker. The fabric was also functionalized with silver zeolites and silver zeolites with chitosan film as a control. The thermoregulatory capabilities of the textiles fabrics were assessed both by differential scanning calorimetry and infrared thermography, which provided information

on the heat distribution over the fabric surface. The bioactive properties were tested both in term antimicrobial activity and silver ions released. The work reported how the different properties were synergic, in facts thermal regulation, bacterial inhibition, and silver ions controlled release was obtained.

8.4.2.2 Textiles for Protection

In the last decades, several efforts have been made to impart functional properties to textile materials in order to make them beneficial to people health (Li et al. 2015; Mostafalu et al. 2017; Yao et al. 2018). In the context of bio-functional garments, a significant amount of studies have been conducted to develop innovative finishing that can protect the human body from potential harms (Bui et al. 2017; Fornasiero 2017). The growth of bacteria and other microorganisms onto the textile surface has been a common issue occurring due to the fact the usage condition of the garments are the ones that usually favors the cell growth of several pathogens (Morais et al. 2016). Besides being a potential threat to human health the growth of microorganism onto textile also lead to a series of side effects such as unpleasant odors, stains and ruining of the materials (Yuan and Cranston 2008). For this reason, several innovative finishing has been developed to impart antimicrobial properties to textile materials. Concerning the possible strategies to inhibit the microbial growth several approaches have been developed; concerning the use of antibiotics, several concerns have aroused in the last years due to the proven capability of several bacteria to evolve and developing antibiotics resistance (Parisi et al. 2017). Therefore great interest was paid in textile technology to substitute the antibiotics drugs with inorganic nanomaterials such as silver, zinc and titanium-based nanoparticles (Bashari et al. 2018). Such materials can release positively charged ions that can interact with the negatively charged bacterial cell wall; this leads to the rupture of the cell wall and the bacterial death (Hoseinzadeh et al. 2017). However, some issues were also aroused concerning the potential toxicity of metal-based nanomaterials and their side effects (Bouwmeester et al. 2018). Chitosan was found to be an exciting alternative to inorganic antimicrobial materials being a natural biocompatible and biodegradable polymer, moreover, its abundance in nature makes it much more convenient from an economic point of view if compared to silver and titanium (Ruocco et al. 2016; Tokatlı and Demirdöven 2018). In the context of using polymeric nanomaterials for antimicrobic application, chitosan has presented the advantage of being intrinsically antimicrobic. Therefore there is no need to chemically modify or conjugate it with drug molecules as it is done with other polymers (Parisi et al. 2017; Verlee et al. 2017). The intrinsic antibacterial activity of this polymer lies in its chemical structure which is rich in amino groups that get protonated in the biological environment; therefore it can easily bind to the microbial cell wall, altering its structure and permeability and inhibiting the DNA replication (Helander et al. 2001; Li et al. 2016). Moreover, chitosan is a very versatile polymer that can be easily chemically modified to enhance its functional properties or employed as a polymeric shell to incorporate various substances in the forms of micro and

nanoparticles (Divya et al. 2017; Wang et al. 2017). Indeed, using chitosan as shell material for micro and nanocarrier production presented several advantages due to the mucoadhesive properties of this polymer, this showed an enhancement of the interactions between the particles and the target tissue improving the release of the encapsulated substance (Ma et al. 2017; Parisi et al. 2017). Moreover, the assembly of chitosan in micro and nanoparticles was proved to the available surface area for cell wall interaction and leads to improvement in the antibacterial properties (Ali et al. 2011; Perelshtein et al. 2013). The application of micro and nanoparticles to textile fabrics is an up-and-coming technology, which has been deeply studied in the recent years to impart functional properties to the commodity garments (Salaün 2016; Massella et al. 2017). The nature of the core material to be encapsulated determines the properties and the application of the produced material; these functional properties of the core substance combined with the antimicrobial properties of chitosan have been giving the chance to produce a new generation of multifunctional garments. Studies in this area have a wide approach that starts from the formulation of the particles and the characterization of their properties to the application of the particles to the textile substrate and the assessment of the properties of the functional textile. Ali et al. (2011) attempted to combine and improve the antimicrobial properties of silver by encapsulating it in chitosan nanoparticles. The particles were produced by an ionic gelation method and applied to polyester fabrics. Given the scarce reactivity of polyester, the textiles were surface activated with a sodium hydroxide pretreatment before applying the nanoparticles by an exhaustion bath treatment carried on for 45 min at 60 °C. The extent of chitosan adsorption on the fabric was assessed by carrying out dyeing with an acid dye (Navy blue), which interacts with the $–NH_3^+$ groups of chitosan. The color intensity measured by spectrophotometry and correlated to the amount of chitosan-grafted. The antimicrobial assay carried onto the textile evidenced how the combination of silver and chitosan in the nanoparticle form delivery better bacterial neutralization for the single materials, evidencing a synergistic antimicrobial effect. In antimicrobial textile functionalization, it is important to take into account that some antibacterial substances agents many cause skin irritation in case of high dosage and uncontrolled release. In the work of Zemljič et al. (2017), the powerful antimicrobial I_2 was incorporated in chitosan nanoparticles to control its release and exploit its antimicrobial and antioxidant activity without irritating side effects. The particles were produced by an ionotropic gelation method and attached to the viscose fibers by immersion and padding protocol. Fibers were previously treated with sodium chlorite to form carboxylic group onto the surface which could better interact with amino groups of chitosan. The nanoparticles functionalized fibers displayed better antimicrobial and antioxidant properties than the ones functionalized by iodine and chitosan alone, showing that the polymer exerts no inhibition of iodine activity. The two species then acted synergistically, and the encapsulation allowed controlling the I_2 dosage, reducing the risk of irritation. The innovative approach of using chitosan to stabilize the phospholipid membrane of liposomes prior to attaching them on textile is presented in the work of Nada et al. (2018). In this case, the molecular chains of chitosan were broken down by oxidative degradation in $NaNO_2$. The depolymerized

chitosan was then used to hydrate the thin phospholipid film during liposome production. The chitosan stabilized liposome incorporated an appositively synthesized Pyrazole active compound, the particle emulsion was then attached to cotton fabrics by padding followed by air drying curing, citric acid was employed as a non-toxic crosslinker. The textile material was investigated for its mechanical properties and biological one by assessing antimicrobial activity and cytotoxicity on skin melanocytes. The fabrics were non-toxic for skin cells and displayed antimicrobial activity. However, no synergistic effects of chitosan and pyrazole were observed, probably due to the degradation of the molecular chains during the pretreatment. A loss of the hand of the cotton was noticed as a consequence of the finishing. The hand loss and in general the alteration of the mechanical properties of the textiles upon the application of the antimicrobial finishing is a factor that could negatively influence the comfort and wearability of the mentioned bio-functional garments. Varan (2017) studied how the chitosan finishing could influence the mechanical properties of nylon/elastane pressure garment for rehabilitation and burn scar management. In this work, chitosan was applied in bulk form, and the parallel test of antibacterial and mechanical properties was conducted to inquire how imparting antibacterial finishing influences the mechanical response of the material. The binding of chitosan with the fibers caused a slight increase in stiffness together with a small decrease in air permeability, bursting strength and drapeability; porosity values instead decreased significantly upon chitosan application. The antimicrobial activity was successfully proven.

In the design of a bio-functional textile it also of crucial importance to assess the durability of the microcapsules treatment, as a matter of facts the washing fastness is a critical parameter in determining the product life. Lam et al. (2013) investigated how multiple washing cycles influence the properties of cotton fabrics functionalized with berberine loaded chitosan microparticles. The study showed that a loss of about 40% of the finishing was occurring in the first 20 washing cycles, while 50 washing cycles were necessary in order to remove the finishing entirely. The antimicrobial activity was maintained for 20 washing cycles; the authors explained this phenomenon by taking into consideration the limited diffusion of the drug from the microparticles.

As discussed the primary interest in producing such innovative textile materials was mainly driven by the goal of producing garments that could protect the user from sources of harm for its health, and beyond the biological risk other potential harms were identified and tackled by the textile technologist. Therefore, significant efforts were made to design protective garments against UV-radiations, insects, and parasites. The usage of chitosan in the production of these textiles has been proved to be a smart way to incorporate the functional substance on the textile while imparting a further antimicrobial effect to the textile garments.

Zinc oxide is versatile that has found application in many fields (Cauda et al. 2014; Laurenti and Cauda 2017), in the context of textile it has been employed to impart antimicrobial properties, UV protection and hydrophobicity (Ashraf et al. 2014; Doumbia et al. 2015). In the work of AbdElhady (2012), the ZnO was incorporated in chitosan nanorods by an ultrasound-assisted method. The nanorod was

applied to cotton fabrics by padding drying and curing protocol. The UV spectroscopy analysis showed a functional capability of the material of acting as UV shield. Furthermore, the material displayed marked antibacterial effect. However, this research did not inquire whether a synergic interaction between the antimicrobial effects of zinc ions and chitosan was occurring which was instead highlighted by Perelshtein et al. (2013). In this work, a novel sonodynamic approach is presented in order to form the chitosan-ZnO nanoparticles directly onto the textile material, without the need of using any binging chemicals. The higher surface area of the obtained particles allowed to boost the antibacterial activity of the raw bulk materials.

Another possible threat for which protective garments could be helpful are insects, as a matter of several insects such as mosquitoes are responsible for the transmission and spreading of infectious diseases like malaria. The encapsulation of insect repellent substances in suitable carriers followed by functionalization of the textile material was, therefore, the adopted strategy to design insect protective garments (Peila et al. 2017). In the work of Souza, the insect repellent limonene oil was encapsulated in chitosan microcapsules by oil in water emulsion technique; capsules were then padded on cellulose non-woven fabric. The release profile of the volatile limonene oil was studied by putting the textile material in a controlled temperature oven and measuring the weight loss at given time intervals. This work marked how the encapsulation in the polymer is crucial in avoiding the burst release of active volatile substance and increase the durability of the insect repellent finish (Souza et al. 2014a). In the study of Fei and Xin (2007), N,N-Diethyl-3-methylbenzamide was encapsulated in chitosan and sprayed on cotton fabrics. The mosquitoes repellency was tested by putting the textile on the arms of volunteers which were then placed in a mosquitoes cage, after a while the number of bites on the person's arms was counted to evaluate the effectiveness textile; antimicrobial activity was tested as well. Good results were found regarding mosquitoes repellency and antimicrobial activity. Hassan and Sunderland (2015) encapsulated dodecyltrimethylammonium chloride in chitosan with the aim of making wool fabrics better withstand the degradation due to microorganism and insects like moths. Before finishing the wool fabrics were attached scoured, and then the microcapsules were applied by a pad-dry-curing process. The finishing treatment was proved to be effective in terms protecting wool from insects and bacteria but had some slighted influences the hand of the fabrics.

To sum up, chitosan-based capsules have been proved to be advantageous materials for protective garments applications. The intrinsic antimicrobial activity of the shell material was generally enhanced when assembled in micro and nanoparticles due to the increase in available surface area, this, combined with the functional properties of the core material allowed the design of improved protective garments. When an antimicrobial substance was incorporated in the particles, an improvement of the overall performance concerning raw materials was observed thanks to a synergic effect. Instead, if core materials with other functionalities were incorporated, it was possible to impart multiple protective effects to the textile garment, such antibacterial and UV protection or antibacterial and insect repellency.

8.5 Conclusions and Future Perspectives

Microencapsulation has been proven to be a versatile technique which can enhance the performance of several substances for a wide range of applications. The research in this field if therefore really active toward the proposal of innovative solutions for bringing to market microcapsules based products. A big focus is being paid nowadays to develop productive encapsulation processes that exploits green chemicals with the aim of making the production easily up scalable. From the formulation point of view it is of great interest to propose methodologies that could display effective loading of the active substance while controlling the release kinetic. In the present chapter the efforts of the scientific community toward the achievement of effective and environmentally friendly microcapsules production evidencing the progress in the field over the last decades and trying to forecast the future trends. Among the various fields in which microcapsules found application a special attention was paid on the production of smart textiles. In this context our review highlighted how significant efforts were made toward the production of durable microcapsule based finishes that could avoid toxic chemicals during the fixation process. Given the strict contact between the smart textile the exploitation bio sourced materials was proposed to be an effective solution in textile finishing. A very interesting trend was then observed in the use of chitosan which was widely exploited both because of its intrinsic antimicrobial activity and good performances as shell material for capsules production. The present work evidenced how this polymer owns peculiar properties that both eases the encapsulation process and control the release kinetics, furthermore the technologies employed to produce chitosan particles deeply explained. Then the analysis focused on how the chitosan capsules can be easily attached to textile substrates evidencing how the chemical nature of this polymers allows effective grafting on textiles surface while avoiding toxic chemicals. A wide range of application of capsules to textile materials was reviewed evidencing how this kind of materials displayed promising experimental results. Such results allow to conclude that the combination of chitosan and micro encapsulation technology could play a determinant role in the design of the garments of future that could satisfy the consumer needs while complying to new regulations in terms of process safety and environmental impact. The way in which textile research opened up to other sectors to provide functional properties to commodity garments will definitely have a significant impact on people life and society in the upcoming years.

Microencapsulation as research axis still has excellent potential for development, particularly in the formulation of more environmentally friendly methods, the choice of the active ingredients to be coated, the formulation of a structuring polymer membrane, or on methods textile finishing for the fixing of the particles and/or the functionalization of the supports. Since the last decade, the main issues are the evolution of legislation in terms of toxicity the products used, the biocompatibility of the raw materials for the textile finishing systems development in response to environmental stimuli (“smart” membranes), the extension of encapsulation meth-

ods for water-soluble active without the use of volatile organic solvent, and the integration of these functional coatings (microcapsule-textile) in other application areas.

In this context, the use of chitosan as well as shell forming material to entrap the active substance and to protect it until the final use, and as antimicrobial compounds represents a wide opening to design new textile materials with added values. Thus, future work will focus not only on chitosan purification in term of molecular mass to better control of the shell formation mechanism but also the textile finishing processes under mild conditions. Therefore, exploiting the potential of chitosan for microencapsulation pass through research, process control, but also by the combination and adaptation of different technology. Research in the textile industry must continue to open up other sectors to develop the textiles of the future desired by the consumer in accordance with the legislation. Nevertheless, the microencapsulation methods using chitosan as shell forming material has a sort synthesis time and is cost-effective.

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